Volume 220, number 1, 223-226 FEB 05005 August 1987

Dimethylallylpyrophosphate:3,9-dihydroxypterocarpan 10-dimethylallyl transferase from *Phaseolus vulgaris*

Identification of the reaction product and properties of the enzyme

David R. Biggs*, Roland Welle, Frans R. Visser° and Hans Grisebach

Lehrstuhl für Biochemie der Pflanzen, Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, FRG

Received 22 June 1987

A microsomal fraction of elicitor-challenged bean (*Phaseolus vulgaris*) cell suspension cultures catalyses the prenylation of 3,9-dihydroxypterocarpan (DHP) to 3,9-dihydroxy-10-dimethylallylpterocarpan (phaseollidin) with dimethylallylpyrophosphate (DMAPP) as the prenyl donor. K_m values for DMAPP and DHP are in the range 1–3 μM. Strong product inhibition with phaseollidin and phaseollin is observed.

Prenyltransferase; Phaseollidin; Cell culture; (*Phaseolus vulgaris*)

1. INTRODUCTION

Isoflavonoids have been shown to be potent feeding deterrents to root-eating larvae of the beetle *Costelytra zealandica* (Coleoptera, Scarabaeidae), an important pasture pest in New Zealand. Pterocarpans containing a prenyl or prenyl-derived substituent, particularly in the D ring, were found to be the most active feeding deterrents [1].

Since white clover (*Trifolium repens* L.) contains only unprenylated isoflavonoids [2], transfer of a prenyltransferase gene to this plant could increase its resistance towards larvae of *C. zealandica*. Towards this goal we report in this paper the preparation of a microsomal fraction from elicitor-challenged cultured bean cells which catalyses the prenylation of 3,9-dihydroxypterocarpan (DHP) at position 10 to form phaseollidin (fig.1).

Correspondence address: H. Grisebach, Lehrstuhl für Biochemie der Pflanzen am Biologischen Institut II der Universität, Schänzlestraße 1, D-7800 Freiburg, FRG

°Biotechnology Division, DSIR, Palmerston North, New Zealand

*On leave from Biotechnology Division, DSIR, New Zealand

2. MATERIALS AND METHODS

2.1. *Materials*

Unlabelled (±)-3,9-dihydroxypterocarpan and dimethylallylpyrophosphate (DMAPP) were prepared as described [3,4]. (±)-[6a,11a^{3}H]DHP was available from previous work [5]. [^{3}H]-DMAPP was synthesized by published methods [6] except that EtOH replaced MeOH in the $NaBH_4$ reduction step, the time for reduction was reduced from 15 h to 30 min, and the whole material (35 μmol) was taken as a single batch through the entire procedure. Phaseollin and phaseollidin were isolated from bean roots by repeated silica gel and LH20 chromatography, and gave UV, MS and ^{1}H-NMR (80 MHz) spectra in agreement with reported data [7,8]. Before use, the compounds were further purified by reverse-phase HPLC (RP18, 10 μm, MeOH/H_2O, 60:40, v/v). (±)-Medicarpin and coumestrol were from our laboratory collection. Other materials were obtained from commercial sources and were the highest grade available.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2.2. *Cell cultures*

Suspension cultures of *Phaseolus vulgaris* obtained from W. Barz (Münster) were grown in the dark at 25°C in Gamborg's B5 medium and subcultured weekly.

2.3. *Enzyme preparation*

Cells from 6-day-old cultures were transferred to fresh medium (8 g fresh wt cells per 40 ml medium) and incubated 12 h in the dark. A sterilised yeast extract (Sigma) was added as elicitor to a final concentration of 0.3% (w/v), and the cultures incubated for a further 12 h. Cells were then harvested, washed briefly in water and chilled. They were macerated in a mortar and pestle, and a microsomal fraction preparedd as described [9]. The pellets were suspended in 0.10 M Tris-HCl, pH 7.5, containing 14 mM mercaptoethanol and 20% sucrose, at 5–7 mg/ml protein, and stored at −70°C.

2.4. *Enzyme assay*

The reaction mixture contained in a final volume of 100 μl: 10.6 μmol Tris-HCl (pH 7.5), 1.3 μmol $MnCl_2$, 3.2 μmol fluoride, 1.4 μmol glutathione, 8 nmol (±)-DHP, 0.36–1.1 nmol [^{3}H]DMAPP and 5–10 μg microsomal protein. Alternatively, 0.1 nmol of (±)-[^{3}H]DHP and 16 nmol of unlabelled DMAPP were used. The DHP in ethanol was pipetted into the assay tubes first and the solvent evaporated in vacuo. Reactions were run for 15 to 30 min at 20°C and terminated by the addition of solid NaCl and 200 μl ethylacetate. The tubes were shaken, the organic phase separated by centrifugation and aliquots were counted in a liquid scintillation counter using toluene:0.5% PPO scintillant. Blanks (no DHP) were included in every assay. Phaseollin and phaseollidin in ethanol were treated as for DHP.

2.5. *Preparation and purification of reaction product*

The enzyme assay was scaled up and was incubated for 5 h. After incubation, the tubes wre centrifuged to remove protein and the supernatants combined and extracted (6×) with *n*-hexane. The extracts were dried, and the product purified by HPLC on silica gel (5 μm, ethylacetate/2-propanol, 91:9, v/v) followed by reverse-phase HPLC (RP18, as above).

2.6. *pH dependence and stability*

A universal buffer was made by mixing equal volumes of 100 mM Mes, Mops and Tris. Aliquots (9 ml) were adjusted to pH values over the range 5.25–9.5 in 0.25 unit increments with HCl or KOH. Aliquots of enzyme were assayed directly in buffer/salt mixtures at the desired pH. For stability tests, aliquots of enzyme were pre-incubated for 30 min at 20°C in buffer/salt mixtures of the appropriate pH, then samples were removed and assayed at the optimum pH (7.5). The concentration of buffer in these assays was of the order of 60 mM.

2.7. *Analytical methods*

Thin-layer chromatography was performed on silica gel plates (Merck, Darmstadt, FRG) with the solvent system (1) toluene/chloroform/acetone (45:25:35, v/v); (2) chloroform/methanol (19:1, v/v).

3. RESULTS

When the microsomal fraction from elicitor-induced bean cell cultures was incubated with (±)-3,9-[^{3}H]dihydroxypterocarpan and DMAPP a new radioactive product was formed which comigrated on silica gel plates in solvents 1 and 2

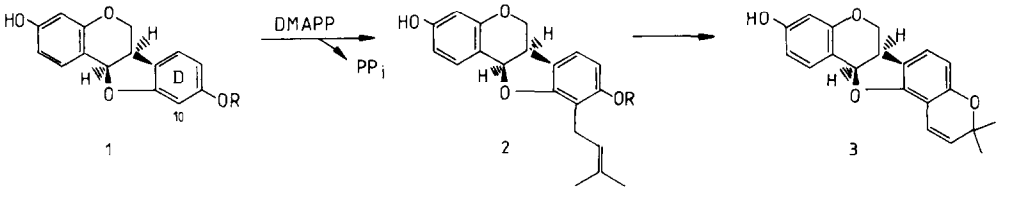

Fig.1. Prenylation of (6a*R*,11a*R*)3,9-dihydroxypterocarpan (**1**, R = H) to phaseollidin (**2**, R = H). The latter is a precursor for phaseollin (**3**). Medicarpin (**1**, R = CH_3).

with phaseollidin. A scaled-up preparation with microsomes from bean cells induced with yeast extract [10] and unlabelled substrates yielded ~200 μg pure product. This was unequivocally identified as phaseollidin by its UV, MS and ^{1}H-NMR spectra [7,8]. The attachment of the dimethylallyl residue at position 10 was shown in the ^{1}H-NMR spectrum by the presence of the signals for H-7 (δ 6.94, J = 8.7 Hz) and H-8 (δ 6.36, J = 8.7 Hz) [8]. The cell cultures were conveniently treated with yeast extract [10] to increase prenyltransferase activity. Synthesis of phaseollidin was also detected with microsomes from bean seedlings which had been treated with 3 mM $CuCl_2$ for 16 h at 20°C.

In the enzyme assay below 30% substrate utilisation, the amount of product formed was proportional to the amount of enzyme added and to the time of incubation. No reaction was observed if either DMAPP or DHP was omitted or if the enzyme preparation was boiled.

The Michaelis constant of the enzyme was determined for DMAPP and (±)-DHP. For both substrates, a plot of $1/V$ vs $1/S$ showed Michaelis-Menten kinetics, with K_m values of 1.5 μM for DMAPP and 2.8 μM for (±)-DHP being recorded.

The enzyme showed maximal activity over the pH range 7–8 (fig.2). At pH values lower than 6.5 the activity was strongly affected, decreasing to zero at pH 5.5. Enzyme stability also showed a marked reduction below pH 6.5, while at higher pH values the stability was little affected.

Phaseollidin, the immediate product of the reaction, inhibited prenyltransferase activity by 50% at

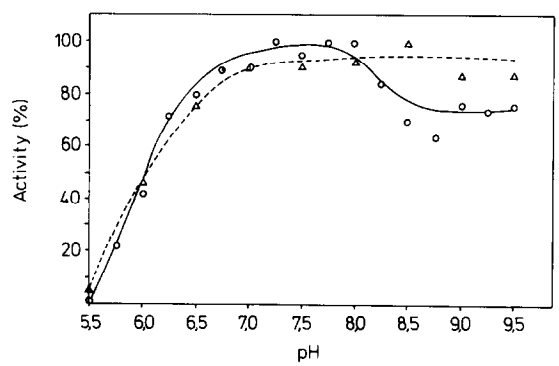

Fig.2. Effect of pH on activity (○—○) and stability (△---△) of prenyltransferase from cultured bean cells.

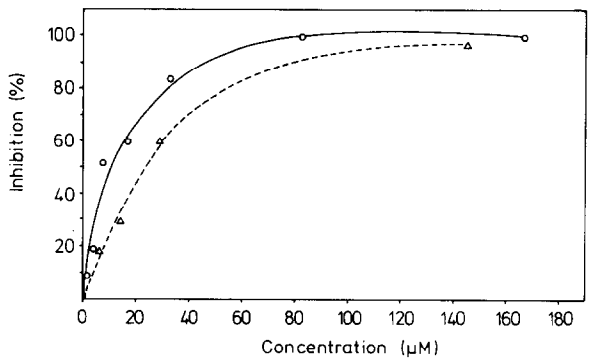

Fig.3. Effect of phaseollidin (○—○) and phaseollin (△---△) on bean prenyltransferase activity. The concentration of (±)-DHP in these assays was 1.9 μM.

8 μM, while phaseollin showed 50% inhibition at 24 μM (fig.3). Reciprocal $1/V$ vs $1/S$ plots gave typical competitive inhibition kinetics for each of these compounds.

The enzyme did not show absolute specificity for DHP. Medicarpin (fig.1) and coumestrol (3,9-dihydroxycoumestan) when tested at saturating concentrations were prenylated at 24% and 27%, respectively, of the rate of DHP. When DMAPP was replaced in the assay by [1-^{14}C]isopentenyl pyrophosphate, incorporation into phaseollidin was reduced by about 90%.

4. DISCUSSION

The prenyltransferase from bean described in this paper is specific for the 10-position of DHP and must therefore be the enzyme involved in the biosynthesis of the bean phytoalexin phaseollin [11,12]. The enzyme has a high affinity for its substrates with K_m constants of the order 1–3 μM. Because we used the racemic DHP whereas only the (−)-enantiomer with (6a*R*,11a*R*)-configuration is expected to be used as substrate [11,12] the true K_m for DHP is about 1.4 μM. The prenyltransferase from soybean which catalyses prenylation in the A ring of 3,6a,9-trihydroxypterocarpan [13,14] gave K_m values of 3.9 μM and 3.6 μM for DMAPP and trihydroxypterocarpan, respectively (unpublished). From the substrates available medicarpin (fig.1, **1**, R = CH_3) and coumestrol were also converted prenylation products which were not identified further. Especially noteworthy is the competitive inhibition of the

bean prenyltransferase by the reaction product phaseollidin, and its further metabolite, phaseollin. In contrast, the soybean prenyltransferase was not inhibited by glyceollidin or by a mixture of glyceollin isomers (unpublished). Solubilization and purification of the bean prenyltransferase are underway.

ACKNOWLEDGEMENTS

The work was supported by DSIR (New Zealand), Bundesministerium für Ernährung, Landwirtschaft und Forsten (FRG) and by Fonds der Chemischen Industrie.

REFERENCES

[1] Lane, G.A., Biggs, D.R., Russell, G.B., Sutherland, O.R.W., Williams, E.M., Maindonald, J.H. and Donnell, D.J. (1985) J. Chem. Ecol. 11, 1713–1735.
[2] Ingham, J.L. (1983) Prog. Chem. Org. Nat. Prod. 43, 1–265.
[3] Visser, F.R. (1987) Aust. J. Chem., in press.
[4] Davisson, V.J., Woodside, A.B. and Poulter, C.D. (1985) Methods Enzymol. 110, 130–144.
[5] Hagmann, M., Heller, W. and Grisebach, H. (1984) Eur. J. Biochem. 142, 127–131.
[6] Davisson, V.J., Zabriskie, T.M. and Poulter, C.D. (1986) Bioorg. Chem. 14, 46–54.
[7] Perrin, D.R., Whittle, C.P. and Batterham, T.J. (1972) Tetrahedron Lett., 1673–1676.
[8] Ingham, J.L. and Markham, K.R. (1980) Phytochemistry 19, 1203–1207.
[9] Kochs, G. and Grisebach, H. (1986) Eur. J. Biochem. 155, 311–318.
[10] Tiemann, K., Hinderer, W. and Barz, W. (1987) FEBS Lett. 213, 324–328.
[11] Woodward, M.D. (1980) Phytochemistry 19, 921–927.
[12] Dewick, P.M. and Steele, M.J. (1982) Phytochemistry 21, 1599–1603.
[13] Zähringer, U., Ebel, J., Mulheirn, L.J., Lyne, R.L. and Grisebach, H. (1979) FEBS Lett. 101, 90–92.
[14] Zähringer, U., Schaller, E. and Grisebach, H. (1981) Z. Naturforsch. 36c, 234–241.