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Isolation of pterocarpan synthase, the terminal enzyme of pterocarpan phytoalexin biosynthesis in cell suspension cultures of *Cicer arietinum*

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Chickpea (*Cicer arietinum* L.) cell suspension cultures transferred into a medium containing yeast extract as elicitor accumulate the phytoalexins medicarpin and maackiain. Concomitant with the accumulation of the pterocarpans, a new enzyme activity is induced which catalyses the conversion of the 2'-hydroxyisoflavanone vestitone to the pterocarpan medicarpin. Maximum enzyme activity was reached 12 h after transfer of cells and then activity declined. The soluble enzyme was partially purified and characterized as pterocarpan synthase.

Pterocarpan synthesis; Medicarpin; Phytoalexin; Pterocarpan synthase; (*Cicer arietinum* L.)

1. INTRODUCTION

Pterocarpan phytoalexins such as medicarpin and maackiain (fig.1) are antimicrobial compounds. They are synthesized by plant species of the Leguminosae in response to stress, which is primarily caused by microbial infection [1–6].

Upon infection with various fungi [7,8] such as the deuteromycete *Ascochyta rabiei* [9], chickpea (*Cicer arietinum* L.) plants accumulate the (6a*R*:11a*R*)-pterocarpans [10] medicarpin and maackiain. Chickpea cell suspension cultures when treated with yeast extract [11,12] or fungal elicitors [13] also synthesize these two phytoalexins in a rapid reaction. The isoflavone formononetin is considered to be a central intermediate in medicarpin and maackiain biosynthesis [14,15].

Recent investigations in this laboratory using yeast-extract-treated chickpea cell suspension cultures succeeded in the isolation and partial characterization of two isoflavone (formononetin) 2'- (and 3'-) hydroxylases [16] and an NADPH:2'-hydroxyisoflavone oxidoreductase [17]; these enzyme reactions are clearly involved in pterocarpan phytoalexin formation [10,12].

This paper describes the successful isolation of a new enzyme activity from chickpea cell suspension cultures, which converts the 2'-hydroxyisoflavanone vestitone (fig.1; R_1 = H; R_2 = OCH_3) to medicarpin as the terminal enzyme reaction in the biosynthesis of this pterocarpan. The pterocarpan

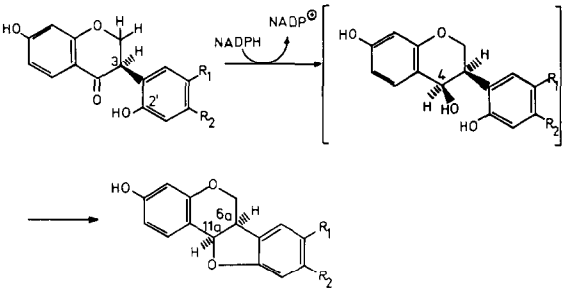

Fig.1. Conversion of 2'-hydroxyisoflavanones (vestitone: R_1 = H; R_2 = OCH_3) to medicarpin (R_1 = H; R_2 = OCH_3) and maackiain (R_1 = R_2: -O-CH_2-O-) as the terminal step in pterocarpan biosynthesis. The possible involvement of an isoflavan-4-ol intermediate [15] remains to be demonstrated.

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synthase was partially characterized with regard to inducibility, cofactor requirement and kinetic properties.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Chickpea cell suspension cultures established from cultivar ILC 3279 were used. The cultures applied for enzyme isolation were grown in a modified PRL-4C medium supplemented with 2.5 g yeast extract/l (Difco, Detroit) as described [11,18]. The cell suspensions were subcultured at 7-day intervals. For enzyme isolation cells were transferred to new medium on day 3 of the growth cycle. The cells were then harvested 12–16 h after elicitation with the yeast extract.

A second chickpea cell suspension line also established from cultivar ILC 3279 has routinely been grown in PRL-4C medium but without yeast extract [10]. Cells of this line inoculated into nutrient media either with or without yeast extract were used for measuring the activity level of the pterocarpan synthase during a growth cycle of the cell culture.

2.2. *Chemicals*

DEAE-Sephacel and Sephadex G-25 (PD-10) columns were purchased from Pharmacia (Freiburg). Polyclar AT, NADPH and NADH were obtained from Serva (Heidelberg). Racemic vestitone was synthesized according to [15]. Enzymatically formed, optically active vestitone was from our earlier investigations [10,17].

2.3. *Buffer systems*

The following buffers were used: Buffer A: 0.02 M potassium phosphate, pH 7.5, with 1 mM dithioerythritol (DTE). Buffer B: 0.2 M potassium phosphate, pH 5.5–7.5. Buffer C: 0.2 M sodium acetate, pH 3.5–5.5. Buffer D: 0.2 M Tris-HCl, pH 8.0–9.5. Buffer E: 0.02 M potassium phosphate, pH 8.0, with 1 mM DTE.

2.4. *Extraction of enzyme activity*

Cells were harvested by filtration and samples (5 g fresh wt) were homogenized with 5 ml buffer A, 0.5 g Polyclar AT and 2 g quartz sand in a mortar at 4°C. The homogenate was centrifuged (20000 × *g* for 15 min) and the supernatant was passed through PD-10 columns using buffer A. The eluate was used directly for enzyme assays.

For preparation of membranes the homogenate was centrifuged (20000 × *g* for 15 min) and the resulting supernatant subjected to ultracentrifugation (100000 × *g* for 60 min). The sediment was resuspended in 2 ml buffer A. Protein was determined according to Bradford [19] using bovine serum albumin as reference.

2.5. *Purification of the pterocarpan synthase*

Suspension cells (30 g fresh wt, harvested 12 h after inoculation into medium with yeast extract) were homogenized in a mortar with 35 ml buffer A and 3 g Polyclar AT. The homogenate was centrifuged (27000 × *g* for 15 min) and the supernatant subjected to ammonium sulfate fractionation. Protein precipitating between 30 and 70% saturation with $(NH_4)_2SO_4$ was collected by centrifugation (30000 × *g* for 30 min), dissolved in 4 ml buffer E and passed through Sephadex PD-10 columns using buffer E. The eluate was chromatographed on a DEAE-Sephacel column (1 × 2.5 cm) equilibrated with buffer E. The proteins were eluted with NaCl in buffer E in batches with 50, 100, 200 and 400 mM NaCl.

2.6. *Enzyme assay*

Enzyme activity was measured in a total volume of 500 μl including buffer B (pH 6), 1 mM NADPH, 0.05 mM substrate (vestitone dissolved in 10 μl methanol was added) and 70 μl enzyme preparation. The reaction was started by the addition of enzyme and incubated at 30°C for 5 min. The reaction was stopped by extraction with 0.8 ml ethyl acetate. The organic layer was recovered, brought to dryness and the residual material was redissolved in 150 μl methanol for HPLC analysis.

For determination of the pH optimum of the enzyme reaction buffer C was used in the pH range 3.5–5.5, buffer B between pH 5.5 and 7.5 and buffer D from pH 8 to 9.5.

2.7. *HPLC analysis*

Determination and quantitation of substrate and product were performed by HPLC procedures as in [9,20] using a linear gradient of 40–85% B (in A) in 20 min. Solvent A was 1.5% H_3PO_4 and solvent B acetonitrile containing 15.9% water. Compounds were detected at 287 nm. The pterocarpan medicarpin appeared in the HPLC chromatograms about 4.8 min after the substrate vestitone.

2.8. *Identification of medicarpin*

Identity of medicarpin as the product of the enzyme reaction was ascertained by HPLC cochromatography with authentic material and UV spectroscopy. The UV spectrum of the isolated product was recorded in ethanol (λ_{max} 227 sh, 283 and 287 nm) [2] or during HPLC with a variable photodiode array detector.

3. RESULTS

Chickpea cell suspension cultures established from cultivar ILC 3279 rapidly accumulate the phytoalexins medicarpin and maackiain when inoculated in yeast extract-containing PRL-4C medium [11,12]. Maximum levels of the pterocarpans are reached 24 h after transfer of the cells (not shown). The yeast extract has been proven to be the elicitor material responsible for pterocarpan accumulation [10,11]. Protein preparations from such elicited chickpea cells contained an enzyme activity which catalysed the conversion of the 2′-hydroxyisoflavanone vestitone to the pterocarpan phytoalexin medicarpin (fig.1). The enzyme reaction was followed by HPLC and the product could unequivocally be identified by cochromatography and UV data [2].

Using ammonium sulfate fractionation (protein fraction obtained between 30 and 70% saturation)

and subsequent DEAE anion-exchange chromatography the enzyme activity was partially enriched. A 3.45-fold purification with a 52% yield of the enzyme activity and 10.5% recovery of protein has been obtained. The partially purified enzyme showed a specific enzyme activity of 166 μkat/kg protein.

The enzyme reaction depends on NADPH. With NADH as cosubstrate only 10% of the enzyme activity obtained with NADPH was measured. The coenzymes FAD and FMN had no influence on the enzyme activity and a combination of the flavin nucleotides together with NADPH led to slight inhibition of enzyme activity. The pterocarpan synthase is a soluble enzyme because only 0.5% of the total enzyme activity was found in a microsomal preparation (100000 × g, 60 min).

The apparent K_m value for vestitone was found to be 1.7×10^{-5} M and for NADPH 4×10^{-5} M. The enzymatic reaction showed a pronounced pH optimum at pH 6 and a temperature optimum of 30°C.

Upon transfer of chickpea cells regularly grown in yeast extract containing PRL-4C medium [11,18] into new elicitor-complemented medium on day 3 of the growth cycle, the enzymatic activity of the pterocarpan synthase showed a drastically transient increase (fig.2). Maximum enzyme activity was found 12 h after transfer of the cells. Suitable controls demonstrated that this pronounced increase in enzyme activity resulted from the elicitor effect of the yeast extract. Thus, in cells inoculated into new medium lacking this elicitor material, only a 30% increase of pterocarpan synthase activity has been measured.

A somewhat different mode of induction of the pterocarpan synthase has been observed in a chickpea cell suspension line routinely grown in yeast-extract-free PRL-4C medium [10]. In this case (fig.3) induction of the pterocarpan synthase activity occurs merely by transfer of cells into new medium and it cannot be increased by addition of elicitor material. Furthermore, maximum enzyme activity was found some 72 h after inoculation of cells on day 3 of the growth cycle. This difference between the two cell culture lines in the expression of pterocarpan synthase activity cannot be explained at the present time.

Chickpea cells produce the (6a*R*:11a*R*)-pterocarpans ([10], fig.1). Preliminary data support the assumption that the pterocarpan synthase distinguishes between the optical isomers of the substrate (fig.1) when racemic vestitone [15] is used. Under assay conditions designed to lead to quantitative conversion of the substrate a maximum yield of only 47% medicarpin could be measured. However, when optically active

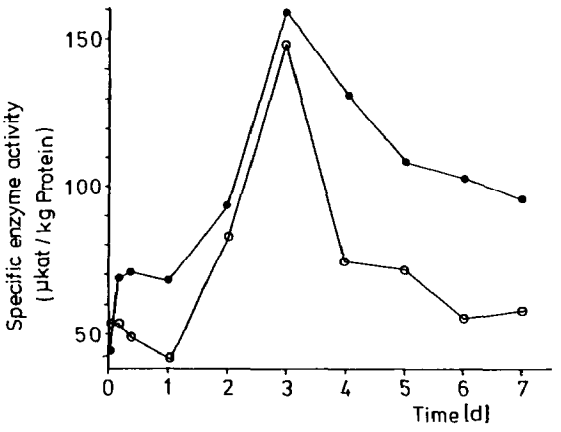

Fig.2. Induction of pterocarpan synthase activity (●—●) upon transfer of chickpea suspension cells from cultivar ILC 3279 into new yeast extract containing medium. Control cells (○—○) were not inoculated into new medium. The maximum of the concomitant accumulation of the phytoalexins medicarpin and maackiain was found 24 h after transfer of the cells.

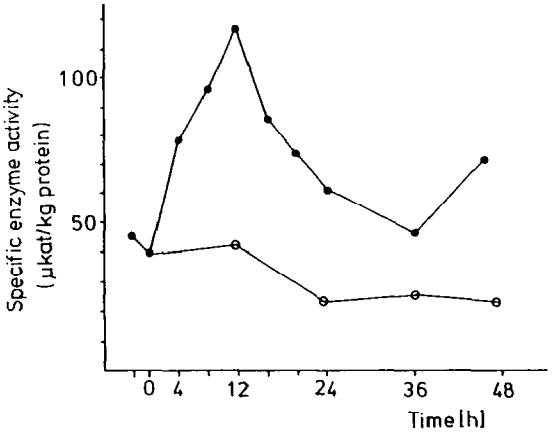

Fig.3. Enzyme activity of pterocarpan synthase during the growth cycle of a chickpea cell suspension culture routinely grown in yeast-extract-free PRL-4C medium [10]. Cells inoculated either into yeast-extract-free (○—○) or yeast-extract-containing (●—●) medium.

vestitone obtained from the NADPH:2′-hydroxyisoflavone oxidoreductase reaction [10,17] is converted under the same optimum conditions up to 70% product could be observed. The missing 30% is probably due to the facile isomerization of isoflavanones at carbon atom 3 [22].

4. DISCUSSION

Treatment of chickpea cell suspension cultures with various biotic elicitors [11,13] or various heavy-metal ions (Jaques and Barz, unpublished) leads to rapid accumulation of the (6a*R*:11a*R*)-pterocarpan phytoalexins medicarpin and maackiain [12]. Concomitant with this induced biosynthesis a new enzyme activity has been found which catalyses the formation of medicarpin from the 2′-hydroxyisoflavanone vestitone (fig.1). The partially purified enzyme has been named pterocarpan synthase.

Appearance of this synthase activity occurs under the same eliciting conditions and with the same transient accumulation kinetics as those of the previously described isoflavone 2′-hydroxylase [12,16] and the NADPH:2′-hydroxyisoflavone oxidoreductase [10,17]. Thus, the latter two enzymes and the pterocarpan synthase appear to act in sequence to convert the central isoflavone intermediate formononetin to medicarpin. The pterocarpan synthase reaction may therefore be regarded as the terminal enzyme of pterocarpan biosynthesis [1,14,15]. In general, the complete biosynthetic pathway of the phytoalexin medicarpin from phenylalanine ammonia lyase to pterocarpan synthase can now be studied in chickpea cell cultures at the enzymatic level [12,22].

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