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Isolation of NADPH:isoflavone oxidoreductase, a new 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 accumulate the phytoalexins medicarpin and maackiain. Concomitant with accumulation of the pterocarpans a new enzyme activity is induced which was characterized as NADPH:isoflavone oxidoreductase. Maximum enzyme activity was reached 16 h after transfer of cells and then activity rapidly declined. The soluble enzyme was partially purified and shown to catalyze the reduction of the isoflavone 2′-hydroxyformononetin to the isoflavanone vestitone which is an intermediate in medicarpin biosynthesis. The enzyme data suggest that 2′-hydroxylation is a prerequisite for the conversion of isoflavones to pterocarpans.

Pterocarpan biosynthesis; Medicarpin; Phytoalexin; Isoflavone oxidoreductase; Vestitone; (*Cicer arietinum* L.)

1. INTRODUCTION

Pterocarpan phytoalexins such as medicarpin and maackiain (fig.2) as well as phaseollin, pisatin and the various isomers of glyceollin are antimicrobial compounds. They are synthesized by plant species of the Leguminosae in response to stress, which is primarily caused by microbial infection [1–5].

Upon infection with *Helminthosporium carbonum* [6], *Nectria haematococca* [7] or *Ascochyta rabiei* [8], chickpea (*Cicer arietinum* L.) plants accumulate the pterocarpans medicarpin and maackiain. Similarly, chickpea cell suspension cultures when treated with yeast extract [9] or fungal elicitors [10] rapidly synthesize these two phytoalexins.

Investigations on medicarpin biosynthesis in $CuCl_2$-treated seedlings of red clover (*Trifolium pratense*) [11,12] suggested that the biosynthetic pathway involves 2′-hydroxylation of the isoflavone formononetin followed by reduction of the 2′-hydroxyisoflavone to the isoflavanone vestitone (fig.2). This biosynthetic sequence has also been postulated for other pterocarpan phytoalexins [12–17].

This paper presents further support for this reduction step by partial characterization of an NADPH:isoflavone oxidoreductase from chickpea cell suspension cultures. Concomitant with pterocarpan formation in yeast extract-treated chickpea cells the transient appearance of the oxidoreductase has been measured. The new oxidoreductase was characterized with regard to the kinetics of its appearance, cofactor requirement and substrate specificity.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Chickpea cell suspension cultures established from cultivar ILC 3279 were used. The cultures ap-

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plied for enzyme isolation were grown in a modified PRL-4C medium supplemented with 2.5 g yeast extract/l (Difco, Detroit) as described [9,21]. The cell suspensions were subcultured at 7-day intervals. To measure the kinetics of enzyme appearance the cells were grown in PRL-4C medium without yeast extract but then inoculated in medium supplemented with the yeast extract.

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). Vestitone and 2′-hydroxyformononetin were synthesized according to [12]. 2′-Hydroxypseudobaptigenin and pseudobaptigenin were gifts from Dr P.M. Dewick (Nottingham). All other isoflavones were from the collection of the institute.

2.3. *Buffer systems*

The following buffers were used: buffer A, 0.1 M potassium phosphate, pH 7.5, containing 1 mM dithioerythritol (DTE); buffer B, 0.02 M potassium phosphate, pH 7.5, containing 1 mM DTE; buffer C, 0.1 M Tris-HCl, pH 8.5; buffer D, 0.05 M potassium phosphate, pH 8.0, containing 1 mM DTE; buffer E, 0.2 M potassium phosphate, pH 5.5–7.5; buffer F, 0.2 M Tris-HCl, pH 7.5–9.5.

2.4. *Extraction of enzyme activity*

Cells were harvested by filtration and samples (5 g fresh wt) were homogenized with 5 ml buffer D, 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 D. The eluate was directly used for enzyme assays.

For the preparation of membranes the homogenate was centrifuged (3000 × *g* for 15 min) and the resulting supernatant subjected to ultracentrifugation (100000 × *g* for 60 min). The sediment was resuspended in 1.2 ml buffer D.

Protein was determined according to Bradford [18] using bovine serum albumin as reference.

2.5. *Purification of the isoflavone reductase*

Suspension cells (300 g fresh wt, harvested 16 h after inoculation into medium with yeast extract) were homogenized in a Waring blender with 350 ml buffer A and 30 g Polyclar AT. The homogenate was centrifuged (27000 × *g* for 15 min) and the supernatant subjected to ammonium sulfate fractionation. Protein precipitating between 40 and 70% saturation with $(NH_4)_2SO_4$ was collected by centrifugation (27000 × *g* for 15 min), dissolved in 15 ml buffer B and passed through Sephadex PD-10 columns using buffer B. The eluate was chromatographed on a DEAE-Sephacel column (3 × 24 cm) equilibrated with buffer B. The proteins were eluted at a flow rate of 90 ml/h using a linear gradient of NaCl (0–0.3 M) in buffer B. Fractions (7.5 ml each) with pronounced isoflavone reductase activity were pooled and stored at −20°C in the presence of 5% glycerol. This material was finally used for enzyme characterization.

2.6. *Enzyme assay*

Enzyme activity was measured in a total volume of 250 μl including buffer D, 1 mM NADPH, 0.1 mM substrate (2′-hydroxyformononetin or 2′-hydroxypseudobaptigenin was added dissolved in 5 μl methanol) and 50 μl enzyme preparation. The reaction was started by the addition of enzyme, incubated at 30°C for 15 min, and stopped by extraction with 1 ml ethyl acetate. The organic layer was recovered, brought to dryness and the residual material redissolved in methanol for HPLC analysis.

For the determination of the pH optimum buffer E was used in the pH range 5.5–7.5 and buffer F from pH 7.5 to 9.5.

2.7. *HPLC analysis*

Determination and quantitation of substrates and products were performed by HPLC procedures as in [8,19] using a linear gradient of 40–45% 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 278 nm. The isoflavanones appeared in the HPLC chromatograms about 2 min before the substrate isoflavone.

2.8. *Identification of vestitone*

The identity of the product was verified by HPLC-cochromatography according to [19] and by UV spectroscopy using authentic reference

Table 1

Partial purification of the NADPH:isoflavone oxidoreductase from *Cicer arietinum* cell suspension cultures ILC 3279

Purification step	Protein (mg)	Spec. act. (μkat/kg protein)	Recovery (%)	Purification (-fold)
Crude extract after Sephadex G-25	381	41.2	100	1
$(NH_4)_2SO_4$ fractionation, 40–70% fraction	182	50.6	58.7	1.2
DEAE-Sephacel	31.8	199	40.3	4.8

material [22] (measured UV spectrum of vestitone: λ_{max} MeOH 212, 230 (sh), 277 and 311 nm).

3. RESULTS

Chickpea cell suspension cultures established from cultivar ILC 3279 rapidly accumulate the phytoalexins medicarpin and maackiain when inoculated into yeast extract-containing PRL-4C medium [9]. 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 [9].

At times of maximum phytoalexin formation enzymatic activity for the reduction of 2′-hydroxyformononetin to vestitone (fig.2) could be extracted from the cells. The enzyme reaction was measured by HPLC procedures and vestitone was identified by UV spectroscopy and cochromatography with authentic reference material.

The enzyme reaction depends on NADPH whereas NADH only led to 25–40% of the enzyme activity with NADPH. The coenzymes FAD and FMN had no influence on the enzyme reaction. The isoflavone reductase is a soluble enzyme. Only 3% of the initial amount of enzyme activity was recovered from the 100000 × *g*, 60 min membrane fraction.

Attempts to purify the reductase were severely hampered by the pronounced instability of the enzyme activity. Without the addition of DTE a complete loss of enzyme activity was observed within 6 h at 4°C. The present purification (table 1) results in a 4.8-fold enriched specific activity with a yield of 40%. This partially purified enzyme was used for enzyme characterization. Apparent K_m values were determined to be 13 ± 2 μM for 2′-hydroxyformononetin and 35 ± 5 μM for NADPH. The enzymatic reduction of 2′-hydroxyformononetin showed an optimum at pH 8.5. The best enzyme activity was measured at 35–40°C. Besides 2′-hydroxyformononetin only 2′,7-dihydroxy-3′,4′-methylenedioxyisoflavone (i.e. 2′-hydroxypseudobaptigenin) served as substrate for the enzyme reaction although the latter substrate

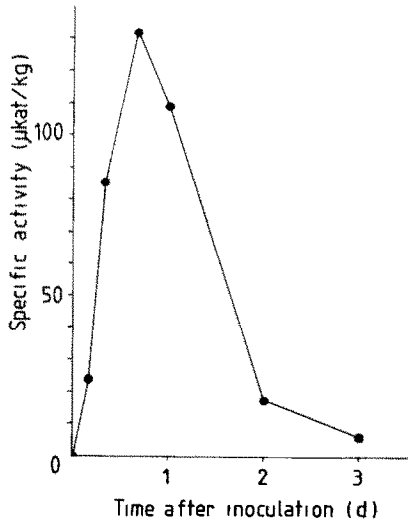

Fig.1. Appearance of NADPH:isoflavone oxidoreductase activity in chickpea cell suspension cultures ILC 3279 after inoculation of cells in yeast extract-containing PRL-4C medium. Control cells were inoculated in medium without yeast extract and then the oxidoreductase activity occasionally rose to 10–20% of the specific activity measured with elicitor.

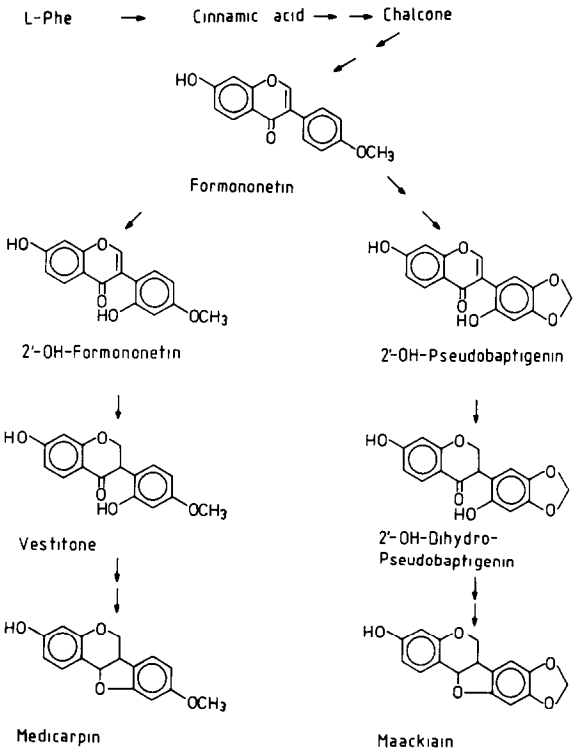

Fig.2. Biosynthetic pathway of the pterocarpan phytoalexins medicarpin and maackiain [1,11,12].

has not yet been further investigated. Isoflavones without a 2'-hydroxy group such as daidzein, genistein, formononetin, biochanin A, pseudobaptigenin and calycosin were not converted to the corresponding isoflavanones.

The appearance of the oxidoreductase activity is restricted to a rather short period after inoculation of cells into fresh yeast extract-containing medium (fig.1). Within 16 h maximum enzyme activity (approx. 130 μkat/kg protein) is reached which rapidly declines and then disappears during the following 48 h. Inoculation of cells into a medium without yeast extract did not lead to any significant levels of the oxidoreductase activity although 'stress factors' due to manipulation of the cells during transfer occasionally led to low levels of enzyme activity.

4. DISCUSSION

Upon treatment with fungal elicitors [10] or yeast extract [9] chickpea cell suspension cultures accumulate the pterocarpan phytoalexins medicarpin and maackiain. Concomitant with phytoalexin formation an enzyme activity has been obtained from such cells which specifically reduces 2'-hydroxyisoflavones (2'-hydroxyformononetin and -pseudobaptigenin) to the corresponding isoflavanones. The reaction depends on $NADPH_2$. In view of the pronounced specificity for coenzyme and 2'-hydroxyisoflavones this new enzyme may be named NADPH:2'-hydroxyisoflavone oxidoreductase. A similar NADPH-dependent enzyme which had been recently purified from the fungus *Fusarium javanicum* [20] shows a pronounced specificity for 5-hydroxyisoflavones with a 4'-methoxy substituent.

All evidence collected so far leads to the assumption that the oxidoreductase is involved in pterocarpan biosynthesis as postulated by Dewick and associates [1,11,12]. The biosynthetic pathway to medicarpin and maackiain (fig.2) based on feeding experiments with suitable intermediates and our data on the oxidoreductase also requires that formononetin and pseudobaptigenin are first hydroxylated in the 2'-position and subsequently reduced to the isoflavanones. The formation of pseudobaptigenin requires a preceding hydroxylation in position 3' [14]. Enzymes catalyzing these hydroxylations have meanwhile been found in our chickpea cells after treatment with yeast extract (Hinderer and Barz, unpublished). Though experimental proof must still be obtained the data in fig.2 support the assumption that an induction process is involved in the accumulation of oxidoreductase activity. This has previously been shown for other enzymes of phytoalexin formation in cell suspension cultures [3,4].

Future investigations on this oxidoreductase should especially deal with a more detailed characterization of the enzyme and the various stereochemical aspects with regard to both NADPH and the chirality of the isoflavanone vestitone. It must also be elucidated whether one or two oxidoreductases are involved in medicarpin and maackiain formation, one for each phytoalexin. Furthermore, the metabolic regulation of the postulated induction mechanism and the integration of this new enzyme in the sequence of other enzymes involved in pterocarpan biosynthesis warrant further experiments.

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