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Microsomal isoflavone 2′- and 3′-hydroxylases from chickpea (*Cicer arietinum* L.) cell suspensions induced for pterocarpan phytoalexin formation

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Microsomal fractions derived from suspension-cultured chickpea (*Cicer arietinum* L.) cells induced for phytoalexin biosynthesis catalyzed the monohydroxylation of 4′-methoxyisoflavones (biochanin A and formononetin) in the 2′- and 3′-positions. The reactions depended on NADPH and molecular oxygen. Post-microsomal supernatants or microsomes from non-induced cells are without detectable activity in the hydroxylase assay. 4′-Hydroxyisoflavones (genistein and daidzein) were not hydroxylated to any significant extent. The occurrence of these hydroxylases proceeds concomitantly with the accumulation of two pterocarpan phytoalexins, medicarpin and maackiain, by induced cell cultures. The results are discussed with regard to the biosynthetic sequences in the conversion of isoflavones to pterocarpans.

Calycosin; 2′-Hydroxyisoflavone; Isoflavone hydroxylase; Isoflavonoid phytoalexin; Pratensein; Pterocarpan synthesis

1. INTRODUCTION

The phytoalexins of chickpea (*Cicer arietinum*) are the pterocarpans medicarpin and maackiain (fig.1) which readily accumulate in infected plants [1–3], in elicitor-treated cotyledons [4] or in cell cultures [5]. The biochemical pathways leading to medicarpin and maackiain were based on feeding experiments with *Trifolium pratense* [6–8] and *Medicago sativa* [9,10]. These investigations suggested that the conversion of isoflavones to pterocarpans (fig.1) involves, firstly, 2′-hydroxylation and subsequently reduction to the corresponding isoflavanones. A 2′-hydroxylation step is common in the formation of all pterocarpans as well as coumestans, rotenoids and isoflavans [11]. 2′-Hydroxyisoflavones and -isoflavanones themselves may function as phytoalexins and accumulate in infected tissues of various Leguminosae [12]. In maackiain biosynthesis a preceding hydroxylation reaction in the 3′-position is required for formation of the methylenedioxy moiety [8] (fig.1).

Recently we presented enzymatic evidence for the reduction step of 2′-hydroxyisoflavones to 2′-hydroxyisoflavanones. A NADPH:isoflavone oxidoreductase which catalyzed the reduction of 2′-hydroxyformononetin and 2′-hydroxypseudobaptigenin (fig.1) was isolated from chickpea cell cultures [13]. This paper presents data on the occurrence and properties of enzymes responsible for the B-ring hydroxylation steps as intermediate reactions in the biosynthesis of pterocarpan phytoalexins in chickpea.

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2. MATERIALS AND METHODS

2.1. *Cell suspension cultures*

Cell cultures of *C. arietinum* L. (cultivar ILC 3279) were grown on modified PRL-4c medium [14] as described [5], except that glycine (2 mg/ml) was substituted for yeast extract. Cells were in-

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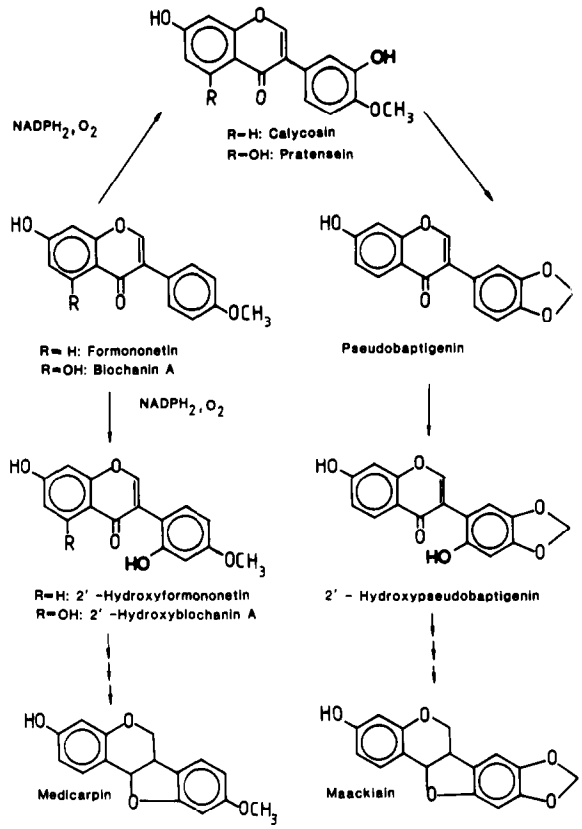

Fig.1. Hydroxylation of 4'-methoxyisoflavones catalyzed by microsomal fractions from chickpea cell cultures. The reactions with formononetin are shown as part of the biosynthetic pathway leading to medicarpin and maackiain [11]. The reactions with biochanin A are not involved in pterocarpan biosynthesis in chickpea.

duced by inoculation of 80 ml cell suspension (approx. 10–15 g fresh wt), 3 days after final transfer, into 400 ml fresh medium (1 l flask) supplemented with 2.5 g/l yeast extract.

2.2. *Enzyme preparation*

Cells were harvested by filtration 16 h after inoculation into yeast extract containing medium and washed with 50 ml buffer A. A 30 g portion of the cells was ground in a mortar together with 10 g Dowex 1X2 (phosphate form), 5 g quartz sand, and 30 ml buffer B. The homogenate was filtered through a 150 μm nylon net and centrifuged at 10000 × g for 10 min. After passing through a 60 μm nylon net the supernatant was further centrifuged at 145000 × g for 60 min. The resulting microsomal pellet was carefully washed with 2 ml buffer B and resuspended in 3 ml buffer B using a Potter-Elvehjem glass homogenizer. The enzyme preparation was either used directly for enzyme assays or frozen and stored at −20°C for at most 24 h.

2.3. *Enzyme assay*

The hydroxylase assay mixture contained in a final volume of 2 ml: 1.65 ml buffer C, 1 mM NADPH (added with 0.5 ml buffer C), 50 μM isoflavone (added with 50 μl methanol) and 0.3 ml enzyme preparation (0.4–0.6 mg protein). The reaction was started by addition of the enzyme and incubated in 10-ml tubes at 25°C for 30 min. In the controls the substrates were omitted. The reaction was stopped by extraction with ethyl acetate (2 × 2.5 ml). After separation of the phases by centrifugation the collected organic layers were evaporated to dryness, redissolved in 1 ml ethyl acetate, transferred to Eppendorf vials and dried again. Before subjecting to HPLC the residues were redissolved in 100 μl methanol.

The experiments with exclusion of oxygen were performed using a modified assay. The assay mixture contained in a final volume of 600 μl in closed Eppendorf vials: 480 μl buffer C, 1 mM NADPH, 50 μM formononetin (added with 20 μl methanol) and an oxygen-consuming system consisting of 30 mM glucose, 1.7 μkat glucose oxidase and 2.3 μkat catalase. All solutions were flushed with nitrogen prior to use. The samples were preincubated for 15 min at 25°C and then started by the addition of the enzyme (100 μl microsomes). After 60 min the reaction was stopped and extracted twice with 500 μl ethyl acetate. The combined organic phases were dried and redissolved in 100 μl methanol before HPLC analysis started.

2.4. *HPLC analysis*

Separation of the substrate and the products was performed by HPLC according to Köster et al. [15]. For quantitation of the products external standardization at 261 nm (for 2'- and 3'-hydroxybiochanin A) or 248 nm (other isoflavones) was applied. The amount of 2'-hydroxybiochanin A was calculated using the absorption factor obtained for pratensein.

2.5. *Protein identification*

Calycosin, 2′-hydroxyformononetin, and pratensein were identified by UV spectroscopy, and HPLC and TLC cochromatography with authentic references. GC-MS analysis was performed with all 2′- and 3′-hydroxylated products of formononetin and biochanin A as in [16]. Spectral data:

2′-Hydroxyformononetin (7,2′-dihydroxy-4′-methoxyisoflavone) – UV, λ_{max} (MeOH) 248 nm; MS, $(TMSi)_2$ derivative, *m/e* 428 [M^+], 413 [$M^+ - 15$], 369, 355, 341, 325, 311, 297, 209, 205, 199, 73.

Calycosin (7,3′-dihydroxy-4′-methoxyisoflavone) – UV, λ_{max} (MeOH) 248 nm; MS, $(TMSi)_2$ derivative, *m/e* 428 [M^+], 413 [$M^+ - 15$], 398, 383, 355, 341, 327, 209, 199, 190, 184, 175, 73.

2′-Hydroxybiochanin A (5,7,2′-trihydroxy-4′-methoxyisoflavone) – UV, λ_{max} (MeOH) 261 nm; MS, $(TMSi)_3$ derivative, *m/e*: 516 [M^+], 501 [$M^+ - 15$], 485, 444, 429, 427, 385, 357, 325, 309, 284, 255, 205, 151, 132, 117, 73.

Pratensein (5,7,3′-trihydroxy-4′-methoxyisoflavone) – UV, λ_{max} (MeOH) 262 nm; MS, $(TMSi)_3$ derivative, *m/e*: 516 [M^+], 501 [$M^+ - 15$], 471, 444, 429, 427, 399, 385, 370, 243, 236, 228, 190, 175, 73.

TLC systems used: silica gel 60 F_{254}; (I) dichloromethane/methanol (15:1, v/v); (II) chloroform/isopropanol (4:1, v/v); (III) *n*-hexane/ethyl acetate/methanol (6:4:1, by vol.).

2.6. *Protein determination*

Protein concentrations were determined according to Bradford [17] with bovine serum albumin as reference.

2.7. *Chemicals*

Pratensein and calycosin were prepared by biotransformation of biochanin A and formononetin with the fungus *Fusarium oxysporum* f.sp. *lycopersici* as in [18]. 2′-Hydroxyformononetin was synthesized according to [7]. Formononetin was purchased from Roth (Karlsruhe) and biochanin A from EGA (Steinheim, FRG). Genistein and daidzein were from the collection of the institute. The following products came from Serva (Heidelberg): Dowex 1X2, NADPH, NADH, and catalase. Cytochrome *c* and glucose oxidase were obtained from Boehringer (Mannheim), FMN and FAD were from Sigma (München). Yeast extract was purchased from Difco (Detroit, USA).

2.8. *Buffers*

The following buffers were used: buffer A, 40 mM potassium phosphate, pH 7.5; buffer B, 100 mM potassium phosphate, pH 7.5 containing 400 mM sucrose and 3.5 mM 2-mercaptoethanol; buffer C, 100 mM potassium phosphate, pH 7.0, containing 400 mM sucrose.

3. RESULTS AND DISCUSSION

3.1. *Induced hydroxylase activities*

Chickpea cell suspension cultures (cultivar ILC 3279) exhibit rapid phytoalexin accumulation upon inoculation of cells in yeast extract containing medium [5]. This simple and effective method was now used to induce cells on a scale sufficient for enzyme isolation. Microsomes prepared 16 h after inoculation of the cells contained appreciable hydroxylase activities with formononetin or biochanin A as substrate, when NADPH was present as cosubstrate. Two products of each substrate could be separated by HPLC (fig.2) and were identified as the 2′- or 3′-hydroxylated isoflavones using chromatographic and GC-MS techniques. Good agreement was obtained with mass spectra reported earlier for TMSi derivatives of 2′- and 3′-hydroxyformononetin [19]. The spectrum of calycosin (3′-hydroxyformononetin) is characterized by the M^+ signal as the base peak,

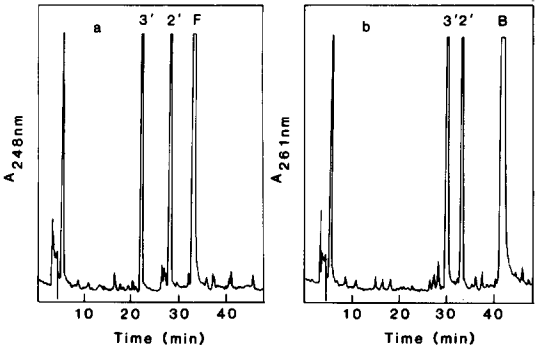

Fig.2. RP-HPLC chromatograms of hydroxylase assays (ethyl acetate extracts) with formononetin (a) or biochanin A (b) as substrate. 2′- and 3′-hydroxylated products could be easily separated from the corresponding substrate.

whereas for the other three isoflavones M^+ is weak and the $M^+ - 15$ ion predominates. No reference was available for 2'-hydroxybiochanin A, nevertheless its identity was deduced from its chromatographic and spectroscopic properties as well as various analogies of the enzyme reaction with the formation of 2'-hydroxyformononetin (not shown). Further products, e.g. 2',3'- or 2',5'-dihydroxylated compounds, have never been observed. Fortunately, no subsequent reduction of 2'-hydroxyformononetin occurred in our assays, due to the localization of isoflavone reductase in the soluble fraction [13]. In some incubations with formononetin, using high enzyme activities, a further product appeared which co-chromatographed with pseudobaptigenin and which probably arises from calycosin (fig.1). So far, this latter reaction has not been further investigated.

No hydroxylase activities could be measured in post-microsomal supernatants. Likewise microsomal fractions derived from control cells (not inoculated into yeast extract containing medium) had no detectable hydroxylase activities. In contrast, inoculation in yeast extract free medium resulted in some 3'-hydroxylase activity only. Together with various other different properties of 2'- and 3'-hydroxylase (not shown) this result suggests that two distinct hydroxylase enzyme systems exist in the chickpea cells.

3.2. *Substrate specificities*

Table 1 shows a comparison of the hydroxylation rates with various isoflavones as substrates. Both hydroxylation reactions depended on a 4'-methoxy substituent in the B-ring. The activities obtained for the 4'-hydroxyisoflavones genistein and daidzein are negligible and were calculated from two very small peaks which appeared in the chromatograms at retention times assumed to be characteristic for hydroxyisoflavones.

3'-Hydroxylation is also an initial reaction in the metabolism of isoflavones by *Fusarium* fungi. The species *F. oxysporum* f.sp. *lycopersici* transiently accumulates pratensein from biochanin A or calycosin from formononetin [18] and such biotransformations were utilized for the preparation of reference compounds. Interestingly, the 4'-hydroxyisoflavones were also very poor substrates for the fungal 3'-hydroxylation in vivo [18]. However, enzymatic studies on isoflavone hydroxylations have not yet been published, except for the formation of 2'-hydroxygenistein with microsomes from soybean cells, which was briefly reported but not further elaborated [20].

Table 1

Hydroxylase activities with various isoflavones as substrate

Isoflavone (0.05 mM)	3'-Hydroxylation (%)	2'-Hydroxylation (%)
Biochanin A	100.0[a]	100.0[b]
Formononetin	41.6	78.1
Genistein	3.2	2.2
Daidzein	2.4	2.0

[a] 100% = 5.9 μkat/kg
[b] 100% = 4.6 μkat/kg

The chickpea hydroxylases converted biochanin A with somewhat higher rates than formononetin, especially in the case of the 3'-hydroxylase (table 1). The 3'-hydroxylated product of biochanin A, pratensein, is a naturally occurring constituent of chickpea [21], whereas 2'-hydroxybiochanin A has so far only been observed in the trunkwood of *Virola* species [21]. The hydroxylase reactions with biochanin A are not involved in the formation of 5-deoxypterocarpans and therefore it seems questionable whether the formation of 2'-hydroxybiochanin A occurs in vivo. In contrast, 2'-hydroxyformononetin is an intermediate in medicarpin biosynthesis [6,7,13] and calycosin is involved in maackiain biosynthesis [8]. Therefore, the hydroxylases from chickpea were further characterized with formononetin as substrate.

3.3. *Cosubstrate requirement*

In table 2 the cosubstrate dependence of the formation of 2'- and 3'-hydroxyformononetin is summarized. The hydroxylases depended on NADPH as the best electron donor. NADH could substitute for NADPH to a low degree only and showed a synergistic effect in the presence of NADPH. The coenzymes FAD and FMN at concentrations of 50 μM had no effect, except for a slight inhibition in combination with either NADPH or NADH.

Molecular oxygen was absolutely essential for both hydroxylations as demonstrated in table 3.

Table 2

Cosubstrate requirement of hydroxylation reactions with formononetin as substrate

Cosubstrate	Concentration (mM)	3′-Hydroxylation (%)	2′-Hydroxylation (%)
NADPH	1.00	100.0[a]	100.0[b]
NADH	1.00	8.5	13.5
NADPH + NADH	1.00 + 1.00	147.3	134.8
FAD	0.05	0.0	0.0
FMN	0.05	0.0	0.0
NADPH + FAD	1.00 + 0.05	100.4	93.1
NADH + FAD	1.00 + 0.05	3.7	8.2
NADPH + FMN	1.00 + 0.05	93.8	90.3
NADH + FMN	1.00 + 0.05	2.9	11.0

[a] 100% = 5.0 μkat/kg
[b] 100% = 3.9 μkat/kg

Table 3

Exclusion of oxygen from the assay and effect of inhibitors on the hydroxylation of formononetin

Condition	Experiment	3′-Hydroxylation (%)	2′-Hydroxylation (%)
Controls	a–c	100.0	100.0
Catalase (2.3 μkat)	a	104.9	106.4
Glucose (30 mM)	a	62.8	79.0
Glucose oxidase (1.7 μkat)	a	56.4	48.0
Catalase + glucose + glucose oxidase	a	4.1	1.2
N_2 flushing	b	5.1	4.0
Cytochrome *c* (10 μM)	c	4.0	13.6
(100 μM)	c	1.1	2.9
KCN (5 mM)	c	105.2	87.1
EDTA (1 mM)	c	99.1	102.2

Specific activities (μkat/kg) of controls were: 3′-hydroxylase: 0.8 (a), 1.2 (b), 1.8 (c); 2′-hydroxylase: 2.3 (a), 2.7 (b), 1.4 (c); control (b) was flushed with air

Exclusion of oxygen from the assay either by the concerted action of glucose oxidase, glucose, and catalase or by flushing with nitrogen prevented the formation of hydroxylated products. KCN (5 mM) and EDTA (1 mM) had no significant influence on the enzyme activities. In contrast, cytochrome *c* at micromolar concentrations strongly inhibited 2′- and 3′-hydroxylase activity. The common properties of the hydroxylases, i.e. microsomal localization, NADPH dependence, cyanide resistance and inhibition by cytochrome *c* suggested that both activities are most likely cytochrome P-450 monooxygenases. In comparison to animals, a small number of inducible cytochromes P-450 have so far been identified in plants. Cinnamic acid 4-hydroxylase [22], isoflavone synthase [20], and a 5-*O*-(4-coumaroyl)shikimate 3′-hydroxylase [23] were induced in elicitor-treated cell cultures, whereas a flavonoid 3′-hydroxylase from parsley was induced by light [24]. It has also been pro-

posed that a 3,9-dihydropterocarpan 6a-hydroxylase, involved in glyceollin biosynthesis, is a cytochrome P-450 monooxygenase [25]. Future studies must reveal whether the chickpea isoflavone hydroxylases are cytochrome P-450 enzymes.

4. CONCLUSIONS

The detection of isoflavone 2′- and 3′-hydroxylase activities in cells induced for phytoalexin accumulation suggests that both enzymes are involved in pterocarpan biosynthesis. The enzymic data, together with those of the previously reported isoflavone reductase [13] confirm the postulated sequences leading to medicarpin and maackiain [11]. In chickpea, the isoflavone daidzein is first methylated in the 4′-position to formononetin prior to further B-ring modifications. In the pathway leading to medicarpin, formononetin is hydroxylated in the 2′-position, whereas in maackiain biosynthesis a 3′-hydroxyl group and, most likely, the 3′,4′-methylenedioxy moiety are formed prior to 2′-hydroxylation. The subsequent sequence from 2′-hydroxypseudobaptigenin to maackiain seems to parallel the reactions from 2′-hydroxyformononetin to medicarpin as depicted in fig.1.

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