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CYTOCHROME P450-DEPENDENT METHYLENEDIOXY BRIDGE FORMATION IN *CICER ARIETINUM*

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Key Word Index—*Cicer arietinum*; Fabaceae; phytoalexin synthesis; cytochrome P450; maackiain; cicerin; calycosin; pratensein.

Abstract—Microsomal preparations from elicited heterotrophic cell cultures of chickpea in the presence of O_2 and NADPH catalyse methylenedioxy bridge formation from pratensein (3′-OH-biochanin A) and calycosin (3′-OH-formononetin). The enzymatic activities are inhibited by CO and various P450 inhibitors and are therefore characterized as cytochrome P450-dependent.

INTRODUCTION

Maackiain ((−)-(6aR,11aR)-3-hydroxy-8,9-methylenedioxypterocarpan) is a phytoalexin of chickpea (*Cicer arietinum*) and numerous other members of the Fabaceae [1, 2]. Upon elicitation by fungal cell wall fragments it is formed in chickpea cell suspension cultures [3]. Maackiain biosynthesis, first elucidated in red clover [4, 5] and well characterized in chickpea [6], proceeds via the constitutively formed isoflavone formononetin (7-hydroxy-4′-methoxyisoflavone). Following cytochrome P450-dependent 3′-hydroxylation yielding calycosin [7] a methylenedioxy bridge is formed (Fig. 1). The resulting pseudobaptigenin is further hydroxylated in the 2′-position before a reduction and ring closure to the pterocarpan structure yield maackiain. For the second major chickpea isoflavone, biochanin A (5,7-hydroxy-4′-methoxyisoflavone), an analogous pathway exists, with the exception of pterocarpan formation [6]. Here, biochanin A is converted to 3′-hydroxy-biochanin A and, subsequently, methylenedioxy bridge formation yields 5-OH-pseudobaptigenin (Fig. 1). This product is 2′-hydroxylated, reduced and methylated leading to the (−)-(3R)-5,7-dihydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavanone cicerin. Cicerin synthesis can also be elicited in chickpea cell suspension cultures [6].

Methylenedioxy bridge functions often occur in isoflavonoids [8], e.g. the pea phytoalexin pisatin. No data on the enzymes responsible are available [9]. In alkaloid biosynthesis methylenedioxy bridge formations have for the first time been characterized as cytochrome P450- dependent [10, 11] and a hypothetical scheme for cytochrome P450 catalysis has been proposed [10].

Our work aims to characterize and purify cytochrome P450 enzymes involved in 2′-OCH_3-isoflavanone and pterocarpan biosynthesis in chickpea [7]. In this paper we present data showing cytochrome P450-dependence of pseudobaptigenin- and 5-hydroxy-pseudobaptigenin formation (Fig. 1). It was also of interest whether one or two enzymes are involved in the formation of these two products, as previously evidence was found that the four different isoflavone hydroxylations are catalysed by at least three different proteins [7].

RESULTS AND DISCUSSION

The substrates used for the detection of the enzymes catalysing the formation of the methylenedioxy rings, namely 3′-hydroxy-formononetin (calycosin) and 3′-hydroxy-biochanin A (pratensein) (Fig. 1), were isolated from the culture fluid of the chickpea pathogen *Ascochyta rabiei* incubated with formononetin or biochanin A, respectively [12].

The enzyme assays contained either crude protein extracts or microsomal preparations of elicited chickpea cells, substrate and various redox cofactors, e.g. FAD, FMN, NAD^+, $NADP^+$, NADH, NADPH. Isoflavones were extracted from the assay mixture with ethyl acetate and analysed by HPLC. Only after incubation of the microsomal fractions with NADPH and substrate could the formation of new compounds be detected; they were isolated by preparative HPLC and, on the basis of UV spectra and co-chromatography with authentic standards in different HPLC separation systems, were identified as pseudobaptigenin and 5-hydroxy-pseudobaptigenin. The respective enzyme activities are tentatively named pseudobaptigenin synthase (Pss) and 5-hydroxy-pseudobaptigenin synthase (5-hydroxy-Pss). Other products, e.g. the respective products of 2′-hydroxylation

	R
3′-Hydroxy-formononetin (= Calycosin)	H
3′-Hydroxy-biochanin A (= Pratensein)	OH

	R
Pseudobaptigenin	H
5′-Hydroxy-pseudobaptigenin	H

Fig. 1. Methylenedioxy bridge formations in chickpea.

of pseudobaptigenin and 5-hydroxy-pseudobaptigenin, were not observed.

Both standard enzyme assays with the microsomal preparations were linear for 2 hr at 12° and for protein concentrations of up to 100 $\mu g\,ml^{-1}$.

The microsomal enzyme activities both displayed a pH optimum at 7.5 and a temperature optimum at 25°. At this temperature, however, the assays were linear for no more than 15 min. Therefore, it was decided to perform incubations at 12°, because this yielded reliably more product and facilitated detection of the two reactions. These enzyme reactions, in comparison with many other plant cytochrome P450 enzymes, are expressed in only low activities [13]. As a consequence of the low incubation temperature, all calculated activities in this study are underestimates.

NADPH is essential for both methylenedioxy bridge formations, whereas NADH (1 mM) led to a turnover of less than 5% of that of NADPH. In contrast to the reports on a number of cytochrome P450 enzymes [14] simultaneous addition of NADPH (1 mM) and NADH (1 mM) did not enhance enzyme activities. An absolute requirement for oxygen of both enzyme reactions was shown by removal of O_2 from the assay mixture by the glucose/glucose oxidase/catalase system [15]. Under these conditions no product was formed. The apparent K_M values for Pss with calycosin as the substrate and for 5-hydroxy-Pss with pratensein as the substrate are 2 μM and 1.2 μM, respectively (Fig. 2). The values for NADPH are 70 μM (Pss) and 25 μM (5-hydroxy-Pss).

Localization of the enzyme activities in the microsomal fraction, O_2- and NADPH-requirement suggested a cytochrome P450 dependence on methylenedioxy bridge formation. Inhibitor studies were carried out to prove this hypothesis. The best-established criterion for P450 involvement is the blue light-reversible inhibition by CO [16]. Incubation of a microsomal preparation in a CO/O_2 (9:1) atmosphere in the dark led to a complete inhibition of Pss and 5-hydroxy-Pss activities which could be partly reversed by blue light (Table 1). In a N_2/O_2 (9:1) atmosphere an inhibition by only 15–24% was observed.

Several established P450-specific inhibitors were tested for their effect on pseudobaptigenin and 5-hydroxy-pseudobaptigenin formation using our previously described assay procedure. The ascertained inhibitory

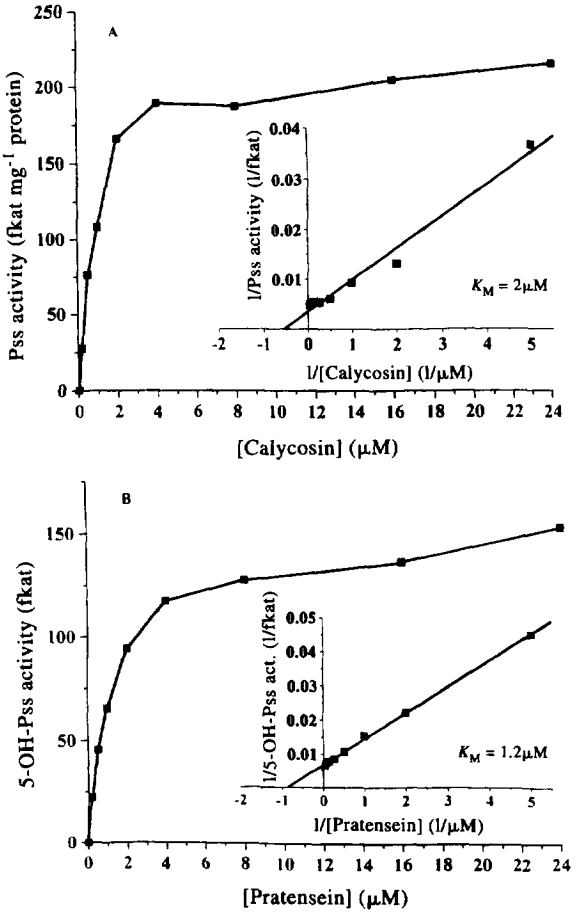

Fig. 2. The effect of calycosin (A) and pratensein (B) concentrations on the methylenedioxy bridge-forming enzyme activities. The insert depicts the double-reciprocal plot.

effects (Table 2) provide further evidence for the P450-dependence of these reactions. Juglone and plumbagin are the most potent inhibitors. The concentrations of cytochrome *c* required for inhibition are unusually high. However, similar values have been reported for the methylenedioxy bridge-forming canadine synthase involved in berberine synthesis [11]. All inhibitors tested led to almost identical degrees of inhibition for Pss and 5-hydroxy-Pss activities. Thus, unlike isoflavone

Table 1. Effect of CO and blue light on Pss- and 5-hydroxy-Pss activities

Assay condition	Enzyme activity (%)	
	Pss	5-OH-Pss
Air, blue light	100	100
N_2/O_2 (9:1), dark	78	76
N_2/O_2 (9:1), blue light	85	79
CO/O_2 (9:1), dark	0	0
CO/O_2 (9:1), blue light	47	53

Standard assay conditions; 100% = 240 fkat Pss and 310 fkat 5-hydroxy-Pss.

Table 2. Concentrations (μM) of cytochrome P450 inhibitors that cause 50% inhibition of Pss- and 5-hydroxy-Pss activities

Cytochrome P450 inhibitor	IC_{50} (μM) for	
	Pss	5-OH-Pss
Tetcyclacis	120	90
Ketoconazole	60	40
BAS 110	400	350
Triadimefone	360	400
Juglone	1	1
Plumbagin	4.5	12
Cytochrome *c*	25	25

hydroxylations in chickpea [7], on the basis of these data Pss- and 5-hydroxy-Pss activities cannot be attributed to different proteins. Solubilization and purification studies including successful reconstitution are required to decide whether one or two enzymes are responsible for pseudobaptigenin and 5-hydroxy-pseudobaptigenin formation. CO difference spectra and data on the cytochrome P450 content and NADPH-P450 (*c*) reductase activities in microsomes from elicited chickpea cells have been published [7].

No synthesis of pseudobaptigenin or 5-OH-pseudobaptigenin can be observed in microsomal fractions from non-elicited chickpea cells in the presence of substrate and NADPH. Treatment of 3-day-old cells with yeast-elicitor [3], however, leads to an induction of Pss- and 5-hydroxy-Pss activities, reaching a maximum 12 hr after addition of the elicitor (Fig. 3). The cytochrome P450-dependent Pss- and 5-hydroxy-Pss enzyme(s) add to the list of inducible enzymes in pterocarpan/isoflavonoid metabolism in chickpea cells [6].

The Pss- and 5-hydroxy-Pss activities, together with the isoflavone hydroxylases of chickpea cells, provide a model system for studies on substrate specificity and regulation of cytochrome P450 enzymes in plants. Six steps in the biosynthetic pathways leading from formononetin and biochanin A to the pterocarpan phytoalexins medicarpin and maackiain and the

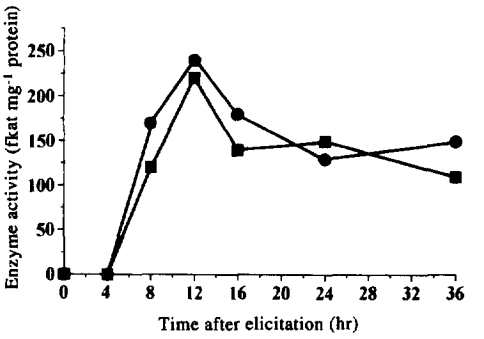

Fig. 3. Pss (●) and 5-hydroxy-Pss (■) activities determined under standard assay conditions in 3-day-old chickpea cells after treatment with yeast elicitor (40 mg/40 ml culture medium). Non-elicited cells failed to express these enzyme activities.

2'-OCH_3-isoflavanones homoferreirin and cicerin, respectively, are now characterized as cytochrome P450 dependent. At least four proteins are, as concluded from inhibitor sensitivities and induction kinetics, responsible for these reactions. Their substrates differ in no more than one hydroxyl group. Determining the exact number of proteins involved requires their separation and reconstitution. Cloning of the genes would enable us to analyse the basis of this high specificity.

EXPERIMENTAL

Substrates. Calycosin and pratensein were prepd via biotransformation of formononetin and biochanin A, respectively, by the fungus *Ascochyta rabiei* [12]. Chickpea meal medium (4% chickpea meal, 3% sucrose) was inoculated with spores (10^5/ml^{-1}) from *A. rabiei*, isolate 21. After 5 days of growth on a rotary shaker at 120 r.p.m. and 20° mycelium was harvested by suction filtration, washed with 0.1 M KPP (pH 7.5) and incubated in this buffer with either formononetin or biochanin A (1 mg ml^{-1}, dissolved in methoxyethanol). After 4 hr (biochanin A) or 24 hr (formononetin) mycelium was harvested again and the KPP extracted twice with 1 vol. of EtOAc. The organic phases were combined, evapd to dryness and the extracted compounds redissolved in MeOH. Calycosin and pratensein were isolated by HPLC using an RP-18 column (125 × 4 mm, 7 μm). The solvent system was MeCN azeotrope/0.15% H_3PO_4. Calycosin and formononetin were sepd by an MeCN azeotrope gradient from 25% to 70% in 12 min (detection at 248 nm), pratensein and biochanin A by a gradient from 40% to 80% in 15 min (detection at 261 nm). Calycosin was obtained in 27% yield, pratensein in 35% yield.

The chickpea cell culture. Derived from cultivar ILC 3279, established in our laboratory and grown as previously described [17]. The cultures were elicited by the addition of 40 mg yeast elicitor/40 ml medium after 72 hr of subculture. The application procedure and the preparation of the yeast elicitor have been reported previously [3].

Microsomal suspensions. Prepared as previously described for chickpea roots [7] with the following alterations: the pH of the homogenization buffer was 8.0 and 2 μM leupeptin was added.

Enzyme assays. Pss- and 5-OH-Pss activities assayed as follows: KPP (50 mM) with 0.4 M sucrose, 1 mM EDTA, 1 mM DTE, NADPH (1 mM), calycosin or pratensein (20 μM) and microsomes (50–100 μg protein) were incubated in a total vol. of 0.5 ml for 2 hr at 12°. The reactions were stopped and the assay mixtures extracted by addition of 0.8 ml EtOAc. HPLC analyses of the products were carried out as described above for formononetin and its derivatives and biochanin A and its derivatives, respectively. The inhibitor studies were performed as previously described [7]. Microsomal protein estimated by the method of ref. [18] using BSA as a standard.

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