Induction and Characterization of a NADPH-Dependent Flavone Synthase from Cell Cultures of Soybean

Georg Kochs and Hans Grisebach

Institut für Biologie II der Universität, Lehrstuhl für Biochemie der Pflanzen, Schänzlestraße 1, D-7800 Freiburg, Bundesrepublik Deutschland

Z. Naturforsch. **42c,** 343–348 (1987); received October 1, 1986

Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Flavonoids, Biosynthesis, Monooxygenase, Cytochrome P-450, Soybean

Microsomal preparations from osmotically stressed soybean cells catalyze the conversion of (2*S*)-naringenin to apigenin in presence of NADPH. In contrast, such preparations from normal soybean cells or from elicitor-challenged cells catalyze the conversion of (2*S*)-naringenin to genistein (isoflavone synthase). It is concluded that osmotic stress of the cells causes a switch from isoflavone to flavone synthesis. The flavone synthase from osmotically stressed cells corresponds in its properties to the microsomal flavone synthase found in several flowers (G. Stotz and G. Forkmann, Z. Naturforsch. **36c**, 737–741 (1981)) and differs from the flavone synthase I from parsley cell cultures which is a soluble Fe^{2+} and 2-oxoglutarate dependent dioxygenase.

Flavone synthase II from soybean has an absolute requirement for NADPH and oxygen. It is inhibited by carbon monoxide in presence of oxygen and this inhibition is reversed by light. It is also inhibited by cytochrome c and by a number of cytochrome P-450 inhibitors. This and other properties show that flavone synthase II is a cytochrome P-450 dependent monooxygenase.

Introduction

The conversion of flavanone to flavone is one of the rare cases in which the reaction has been reported to be catalysed by two different enzyme systems. A soluble enzyme preparation from irradiated cell suspension cultures of parsley catalyzed the conversion of flavanone to flavone [1]. This reaction required 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors and the enzyme was therefore classified as a 2-oxoglutarate dependent dioxygenase (EC 1.14.11). In the meantime this enzyme has been partially purified and shown to be different from flavonol synthase [2]. In contrast, an enzyme preparation from flowers of *Anthirrhinum majus* for the conversion of flavanone to flavone was localized in the microsomal fraction and required NADPH as cofactor [3]. No flavone formation was observed with 2-oxoglutarate, Fe^{2+} and ascorbate. The NADPH-dependent microsomal flavone synthase (here called flavone synthase II) was also found in flower extracts of *Verbena hybrida* and *Taraxacum officinale* [3]. According to its cofactor requirement and microsomal localization it was suggested that this enzyme belongs to the monooxygenases. We now report that the microsomal fraction from osmotically stressed soybean cell cultures catalyzes a NADPH and oxygen dependent conversion of (2*S*)-naringenin to apigenin. This enzyme system, which has the properties of flavone synthase II, has been characterized as a cytochrome P-450 dependent monooxygenase.

Reprint requests to Prof. Dr. H. Grisebach.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0400–0343 $ 01.30/0

Materials and Methods

Chemicals

Flavonoids were from C. Roth (Karlsruhe). (2*S*)-Naringenin was a gift from W. Heller. Elicitor from *Phytophthora megasperma* f. sp. *glycinea (Pmg* elicitor) was prepared as described [4]. The elicitor from *Alternaria carthami* (*Ac*-elicitor) was a gift from H. Strasser (Freiburg). NAD(P)H: FMN-oxidoreductase (EC 1.6.8.1), glucose oxydase (EC 1.1.3.4), catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1) were from Boehringer (Mannheim) and ferredoxin: $NADP^+$-reductase (EC 1.18.1.2) was from Sigma (Deisenhofen).

(2*S*)-[^{14}C]Naringenin (2.34 GBq/mmol) and (2*R*)-[^{14}C]naringenin were obtained as described [5]. (2*S*)-[^{14}C]Eriodictyol was obtained from L. Britsch (Freiburg) and (2*R*, 3*R*)-[^{14}C]dihydrokaempferol from D. Fischer (Freiburg).

Cell cultures and induction

Cell suspension cultures of soybean (*Glycine max* cv. Harosoy 63) were propagated as described [7]

and were transferred every 7th day to fresh medium. For induction experiments 6 g of cells (wet weight) were transferred to 40 ml of fresh medium after 6 days and were then incubated with 0.4 M glucose or with 2.5 mg *Pmg* elicitor (500 µg glucose equivalents). Cells were harvested 10–25 h after induction and were frozen in liquid nitrogen.

Chromatography

For thin-layer chromatography on cellulose (E. Merck, Darmstadt) the following solvent systems were used (ratios by volume): (1) 15% acetic acid; (2) chloroform/acetic acid/water (10:9:1); (3) benzene/acetic acid/water (125:72:3); and on silica gel 60 F_{254} (E. Merck) solvent system (3) and (4) petrol ether/ether (1:1).

Flavonoids were detected under ultraviolet light or by spraying the plates with a 0.2% solution of fast blue B salt (Merck) and fuming with ammonia. Radioactive substances were detected with a TLC-analyzer (Berthold, Wildbad).

Buffers

Buffer A: 80 mM K-phosphate, pH 7.5; buffer B: as A but with 10% sucrose and 14 mM mercaptoethanol; buffer C: 100 mM glycine, 100 mM NaCl, 0.1 N NaOH, pH 7.5.

Preparation of microsomal fraction

The frozen cells were worked up and microsomes obtained by ultracentrifugation (160000 × *g*) as described [1, 5].

Microsomes were suspended in buffer B (5–10 mg protein/ml) and stored at −70 °C.

Assay for flavone synthase II

The assay system contained in 100 µl total volume: 8 µmol buffer A, 72 pmol (2*S*)-[^{14}C]naringenin (10^4 counts/min), 50 µg protein (microsomal pellet) and 100 nmol NADPH. The assay without NADPH was preincubated for 5 min at 10 °C and was started by addition of NADPH. Incubation was carried out for 15 min at 10 °C with shaking in open vials. The reaction was terminated by addition of 50 µl ethyl acetate containing 10 µg each of naringenin and apigenin. The reaction mixture was subsequently extracted twice with each 50 µl ethyl acetate in an ultrasonic bath. The extract was applied to a cellulose plate, which was developed with solvent system 1.

Other analytical methods

The assay of isoflavone synthase [5] and cinnamate 4-hydroxylase [7] has been described. Protein was determined by a modified Lowry procedure [8] after precipitation of the protein with trichloroacetic acid in presence of deoxycholate [9].

Results

Induction of isoflavone synthase and flavone synthase II

Challenge of soybean cell cultures with elicitor preparations from either *Phytophthora megasperma* f. sp. *glycinea* or *Alternaria carthami* increased activity of isoflavone synthase, an enzyme system that catalyzes conversion of (2*S*)-naringenin to genistein in presence of NADPH and oxygen (Fig. 1) [5]. Since it had been found by Ebel *et al.* [6] that osmotic stress of soybean cells caused accumulation of isoflavonoids, we investigated the possibility of replacing the elicitor by an osmoticum for the induction of

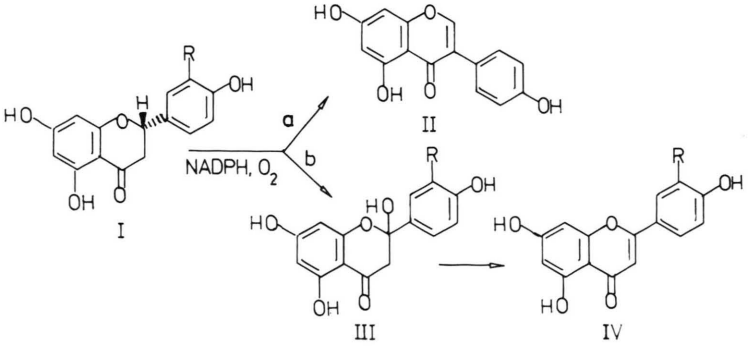

Fig. 1. Conversion of (2*S*)-naringenin (**I**, R = H) by isoflavone synthase (a) to genistein (**II**) and by flavone synthase II to apigenin (**IV**, R = H) *via* the hypothetical 2-hydroxyflavanone (**III**). Eriodictyol (**I**, R = OH) is converted by flavone synthase II to luteolin (**IV**, R = OH).

Table I. Induction of flavone synthase II and isoflavone synthase by elicitor or osmoticum in soybean cell suspension cultures.

Additions to medium	Flavone synthase II [nkat/kg]	Isoflavone synthase [nkat/kg]
None	0	57.7
2.5 mg *Pmg* Elicitor	0	177.3
2.5 mg *Ac* Elicitor	0	127.3
0.4 M Sucrose	269.0	22.3
0.4 M Glucose	387.8	22.8
0.2 M Mannitol + 0.2 M Sorbitol	240.0	12.0

6 g (wet weight) of cells (6 days old) were incubated for 19 h in 40 ml fresh medium.

isoflavone synthase. However, isoflavone synthase activity decreased upon treatment of the cells with either 0.4 M sucrose or 0.4 M glucose or with 0.2 M mannitol plus 0.2 M sorbitol (Table I).

In the enzyme assays with microsomes from cells treated with osmoticum a new radioactive product with an R_f of 0.09 on cellulose with solvent system 1 was found which comigrated with apigenin (naringenin $R_f = 0.58$). This product was identified as apigenin (Fig. 1) by cochromatography with authentic apigenin on cellulose with solvent systems 2 and 3 and on silica gel with solvent systems 3 and 4.

No formation of apigenin was observed with microsomal preparations from either normal or elicitor-treated cell cultures (Table I).

The induction kinetics of apigenin synthesis (flavone synthase II) and the induction of several other enzymes by either elicitor or by osmoticum will be published elsewhere.

It was evident from these results that osmotic stress induces a new enzyme in soybean cells which resembles the flavone synthase previously found in flowers [3].

Some properties of flavone synthase II

Localization

Conversion of naringenin to apigenin in presence of NADPH was only observed in the $160000 \times g$ microsomal pellet from osmotically stressed soybean cells. The supernatant was devoid of activity. The cytochrome P-450 content of the microsomal fraction was determined by a CO-difference spectrum to be 0.65 μmol/l (0.09 nmol/mg protein).

Stability

Enzyme activity was rather unstable. Some stabilization was achieved by addition of 14 mM mercaptoethanol and 10% sucrose (buffer B). The half-lives of enzyme activity in presence of these compounds at 2 °C, 10 °C, 20 °C and 30 °C was 180 min, 110 min, 57 min and 35 min. In presence of mercaptoethanol and sucrose microsomes could be stored at −70 °C for several months without loss of enzyme activity.

Assay for flavone synthase II

The standard assay was carried out at 10 °C because of the low enzyme stability at 30 °C. With 1 mM NADPH and suboptimal concentration of (2*S*)-[^{14}C]naringenin (0.06 K_m) the assay was linear with time up to 40 min and with respect to protein up to 44 μg.

pH Optimum

In potassium phosphate (buffer A) and glycine (buffer C) buffers the enzyme had a narrow pH-optimum of pH 7.5 with half-maximal activity at pH 7.0 and 8.4.

Cofactor dependence

NADPH was essential for flavone synthase II activity and could not be replaced by NADH. As shown in Table II, Fe^{2+}, 2-oxoglutarate and ascor-

Table II. Cofactor dependence of flavone synthase II from soybean.

Addition to assay	S_{160}[a]	Relative activity [%]
NADPH 1 mM	−	100
NADH 1 mM	−	0
Fe^{2+} 50 μM, 2-oxoglutarate 250 μM, ascorbate 5 mM	+	0
FMN 5 μM, NADH 1 mM, FMN oxidoreductase	−	0
6,7-Dimethyl-5,6,7,8-tetrahydroxypterine 1 mM, NADPH 1 mM	−	84
Ferredoxin 10 μg, ferredoxin: $NADPH^+$-reductase, NADH 1 mM	−	7

[a] $160000 \times g$ supernatant.

Table III. Flavone synthase II and cinnamate 4-hydroxylase activity after removal of oxygen with glucose/glucose oxidase.

Addition	Flavone synthase II [%]	Cinnamate 4-hydroxylase [%]
None	100	100
50 mM glucose + 5 U glucose oxidase + 10 U catalase	10	11
50 mM glucose + boiled glucose oxidase + catalase	100	90
10 U catalase + 5 U superoxid-dismutase	100	100

bate, which are the cofactors of flavone synthase I, were completely inactive. The other potential cofactors listed in Table II could also not replace NADPH.

Dependence of the reaction on oxygen was shown by addition of an oxygen consuming system to the enzyme assay which caused a strong decrease in apigenin formation. As a control the inhibition of cinnamate 4-hydroxylase was determined (Table III). Catalase, superoxide dismutase and glucose oxidase did not have a direct influence on enzyme activity.

Substrate specificity

(2*S*)-Naringenin and (2*S*)-eriodictyol were converted to apigenin and luteolin, respectively (Fig. 1). (2*R*)-Naringenin and (2*R*,3*R*)-dihydrokaempferol did not function as substrates. In contrast to the reaction with isoflavone synthase, the conversion of (2*S*)-naringenin by flavone synthase II was not inhibited by presence of an equimolar amount of (2*R*)-naringenin.

The dependence of the reaction rate on (2*S*)-naringenin and NADPH concentration showed Michaelis-Menten kinetics. Apparent K_m values were determined to be 11.4 μM for (2*S*)-naringenin and 39 μM for NADPH, respectively.

Experiments to investigate the participation of cytochrome P-450

Addition of cytochrome *c*, which interferes with the electron transport from the NADPH-cytochrome P-450 reductase to the terminal oxidase [10], inhibited both flavone synthase II and cinnamate 4-hydroxylase. Cytochrome *c* (25 μM) inhibited flavone synthase II activity 47% and cinnamate 4-hydroxylase activity 81%. Addition of 50 μM cytochrome *c* resulted in 72% and 91% inhibition of activity, respectively.

Table IV lists the results of CO-inhibition experiments. Flavone synthase II was strongly inhibited by CO in presence of oxygen and this inhibition could be reversed by white light.

The effect of $NADP^+$, a competitive inhibitor for NADPH-cytochrome P-450 reductase [15], on flavone synthase II is shown in Table V. The results demonstrate that the degree of inhibition depends on the NADPH/$NADP^+$ ratio.

A number of known inhibitors for cytochrome P-450 enzymes [11, 12] were also tested and their effect on flavone synthase II compared with the inhibition of cinnamate 4-hydroxylase. The results listed in Table VI show pronounced differences in the inhibition of the two enzymes. For example, ketoconazole at 1 μM inhibited flavone synthase II 87% whereas no inhibition was observed at this concentration for cinnamate 4-hydroxylase. Keto-

Table IV. CO inhibition experiments.

Composition of gas atmosphere	Flavone synthase II [%]	Cinnamate 4-hydroxylase [%]
O_2/N_2 (10:90)	100	100
O_2/CO (10:90)	7	8
O_2/N_2 (10:90) + light[a]	100	100
O_2/CO + light	88	72

[a] The light source was a slide projector.

Table V. Effect of NADPH/$NADP^+$ ratio on flavone synthase II.

NADPH [μM]	$NADP^+$ [μM]	NADPH/$NADP^+$	Inhibition on flavone synthase [%]
200	200	1	69[a]
100	100	1	71
200	400	0.5	50
100	200	0.5	49
100	400	0.25	47
50	200	0.25	47

[a] Inhibition in relation to enzyme activity without $NADP^+$.

Table VI. Effect of inhibitors for cytochrome P-450 enzymes on flavone synthase II and cinnamate 4-hydroxylase.

Inhibitor	Concentration [μM]	Inhibition flavone synthase [%]	Inhibition cinnamate 4-hydroxylase [%]
None	–	0[a]	0
SKF 525A	100	35	0
	1000	83	15
Ketoconazole	1	87	0
	10	100	12
	100	100	64
Metyrapone	1	11	0
	10	25	7
	100	79	24
Ancymidol	1	59	0
	10	100	26
	100	100	69
Tetcyclacis	1	0	0
	10	15	16
	100	33	36
LAB 150978	1	35	0
	10	80	17
	100	100	28
BAS 110 W	1	28	0
	10	32	0
	100	81	16
KSCN	10 mM	21	28
	100 mM	41	70
	1000 mM	84	95

[a] Specific activity of the controls was 690 nkat/kg for flavone synthase II and 21.1 μkat/kg for cinnamate 4-hydroxylase.

conazole was the strongest inhibitor for flavone synthase II, followed by ancymidol, LAB 150978 and BAS 110 W. The latter two substances are triazole growth regulators from BASF [13]. In general cinnamate 4-hydroxylase was less sensitive to inhibition than flavone synthase II.

Potassium rhodanide, which causes conversion of cytochrome P-450 to cytochrome P-420 [14], inhibited both enzymes to about the same extent.

Discussion

The unexpected and surprising result of the induction experiments is the finding that osmotic stress of the soybean cells causes appearance of a new enzyme activity which is completely absent in the normally grown cell cultures and in the elicitor-challenged cells.

The differential effects of elicitor-challenge or osmotic stress on activities of a number of enzymes will be subject to further investigations. The apparent slight decrease of isoflavone synthase activity observed in presence of osmoticum (Table I) is probably due to the consumption of the substrate naringenin for apigenin formation. The enzyme which is induced by osmoticum is similar to flavone synthase II previously found in the microsomal fraction of several flowers [3].

The enzyme has now been shown to have the typical properties of a cytochrome P-450 dependent monooxygenase. The participation of cytochrome P-450 in the reaction was demonstrated by the following: CO inhibition and its reversion by light; the strong inhibition by typical cytochrome P-450 inhibitors; inhibition by potassium isothiocyanate; the correlation with the inhibition of NADPH-cytochrome (cytochrome P-450) reductase.

A 2-hydroxyflavanone was postulated as an reaction intermediate in flavone synthesis catalyzed by flavone synthase I from parsley cell cultures [1]. The 2-hydroxyflavanone could give flavone by either spontaneous or enzymatic loss of water (Fig. 1). The fact that flavone synthase II is a monooxygenase can also be rationalized by assuming the formation of 2-hydroxyflavanone as the first reaction product. Experiments are in progress to test this hypothesis.

It would also be interesting to know whether the distribution of flavone synthase I or II in the plant kingdom is of taxonomic significance.

Acknowledgements

The work was supported by Deutsche Forschungsgemeinschaft (SFB 206), by Fonds der Chemischen Industrie and by BASF (Ludwigshafen).

[1] L. Britsch, W. Heller, and H. Grisebach, Z. Naturforsch. **36c,** 742–750 (1981).
[2] L. Britsch, Doctoral thesis, University of Freiburg 1986.
[3] G. Stotz and G. Forkmann, Z. Naturforsch. **36c,** 737–741 (1981).
[4] A. R. Ayers, J. Ebel, B. Valent, and P. Albersheim, Plant Physiol. **57,** 760–765 (1976).
[5] G. Kochs and H. Grisebach, Eur. J. Biochem. **155,** 311–318 (1986).
[6] H. Mieth, V. Speth, and J. Ebel, Z. Naturforsch. **41c,** 193–201 (1986).
[7] M. L. Hagmann, W. Heller, and H. Grisebach, Eur. J. Biochem. **142,** 127–131 (1984).
[8] H. Sandermann and J. L. Strominger, J. Biol. Chem. **247,** 5123–5131 (1972).
[9] A. Bensadoun and D. Weinstein, Anal. Biochem. **70,** 241–250 (1976).
[10] M. Fujita, K. Oba, and I. Uritani, Plant Physiol. **70,** 573–578 (1982).
[11] C. J. Coulson, D. J. King, and A. Wiseman, Trends Biochem. Sci. **10,** 446–449 (1984).
[12] J. E. Graebe, Ber. dtsch. bot. Ges. **97,** 67–74 (1984).
[13] J. Jung, C. Rentzea, and W. Rademacher, J. Plant Growth Regul. **4,** 181–188 (1986).
[14] Y. Imai and R. Sato, Eur. J. Biochem. **1,** 419–426 (1967).
[15] K. M. Madyastha and C. J. Coscia, J. Biol. Chem. **254,** 2419–2427 (1979).