Enzymatic rearrangement of flavanone to isoflavone*

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A microsomal preparation from elicitor-challenged soybean cell suspension cultures catalyzes an NADPH-dependent and dioxygen-dependent rearrangement of (2*S*)-naringenin to genistein. The conversion of (2*S*)-5,4′-dihydroxyflavanone to daidzein was also catalyzed by this preparation. The 'isoflavone synthase' was found in elicitor-challenged soybean seedlings as well.

Biosynthesis *Isoflavone* *Monooxygenase* *Soybean* Glycine max *Cell culture*

1. INTRODUCTION

The isoflavonoids are an important subgroup of the flavonoids with interesting biological properties [1]. Many phytoalexins are isoflavonoids [2]. Detailed studies with labelled precursors had shown that isoflavones originate from a chalcone/flavanone precursor by a 2,3-aryl migration [3,4]. With a cell-free extract from *Cicer arietinum* a very low but significant incorporation of [4,4′,6′-^{14}C]trihydroxychalcone 4′-*O*-glucoside into 7-hydroxy-4′-methoxyisoflavone was found, but the enzymes catalyzing these reactions could not be characterized [5]. We now report for the first time on the enzymatic conversion of (2*S*)-naringenin to genistein (see fig.1) with a microsomal preparation from elicitor-challenged soybean cell suspension cultures. Unexpectedly, the cofactors for this reaction proved to be NADPH and dioxygen.

* Dedicated to Professor Karl Decker on the occasion of his 60th birthday

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Abbreviations: TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; HPLC, high-pressure liquid chromatography; *S*-adoMet, *S*-adenosylmethionine

2. MATERIALS AND METHODS

2.1. *Chemicals*

[2-^{14}C]Malonyl-CoA (2.18 GBq/mmol) was from Amersham–Buchler. (2*S*)-[5,4-^{14}C]Dihydroxyflavanone (0.053 GBq/mmol) and other flavonoids were from our collection. Unlabelled (*S*)-naringenin was a gift from W. Heller, Freiburg.

2.2. *Labelled substrates*

(2*S*)-[4a,6,8-^{14}C]Naringenin (2.34 GBq/mmol) was synthesized with an enzyme preparation from parsley cell cultures [7,8].

2.3. *Plant material*

Propagation of soybean cell cultures (*Glycine max* L. cv. Harosoy 63) and treatment with glucan elicitor from *Phytophthora megasperma* f.sp. *glycinea* was carried out as described previously [6]. Five-day-old seedlings of soybean (c.v. Harosoy 63) were treated with elicitor as described [6].

2.4. *Chromatography*

For thin-layer chromatography the following solvent systems were used (ratios by volume): (1) 15% aceetic acid; (2) benzene/acetic acid/water (125:72:3), (3) benzene/acetic acid (7:3); (4) benzene/dioxane/acetic acid (50:50:1); (5) chloroform/acetone/25% ammonia (90:10:5).

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Cellulose plates (Merck, Darmstadt) were developed in solvent systems 1 and 2 and silica gel plates (Merck, 60 F254) or nanoplates (Macherey and Nagel, Sil-20 UV 254) in solvent systems 2–4. Flavonoids were detected under UV-light (254 nm) or by spraying the plates with a 0.2% solution of fast blue B salt (Merck) followed by fuming with ammonia.

HPLC was performed on Partisil 5 (Merck) with hexane/isopropanol (92:8); retention times (min): naringenin 16.5; genistein 15.0. Reversed phase HPLC was carried out on Lichrosorb RP18 (Merck) under isocratic conditions with methanol/water (55:45); retention times (min): naringenin 13, genistein 15.8.

2.5. *Buffer*

The following buffers were used: (A) 0.2 mol/l Tris–HCl (pH 7.5), 14 mmol/l mercaptoethanol, 20% sucrose; (B) 0.08 mol/l K-phosphate (pH 7.5), 14 mmol/l mercaptoethanol, 20% sucrose; (C) 0.1 mol/l Tris–HCl (pH 7.5), 28 mmol/l mercaptoethanol; (D) 0.5 mol/l glycine–NaOH (pH 8.8).

2.6. *Preparation of microsomes*

Elicitor-treated soybean cell cultures were harvested and a cell extract prepared in buffer A as described [6]. After centrifugation of the filtered extract (40 μm mesh nylon) at 100000 × g for 80 min the microsomal pellet was dissolved in buffer B and homogenized in a potter homogenizer.

2.7. *Assay for isoflavone synthase*

The incubation mixture contained in a total volume of 100 μl: 5.6 μmol buffer B, 75 pmol (2*S*)-[^{14}C]naringenin (10^4 cpm), 100 nmol NADPH and 10–15 μg protein. Incubation was carried out for 15 min at 30°C with shaking in open vials. The reaction was terminated by addition of 5 μl naringenin solution (1 mg/ml methanol). Subsequently the mixture was extracted twice with each 50 μl ethylacetate. The extract was applied to a cellulose plate which was developed with solvent 1 and was then scanned for radioactivity (TLC analyzer, Berthold, Wildbad). Enzyme activity was determined by integration of the peak areas of genistein and naringenin.

2.8. *Methyltransferase preparation*

Four-day-old seedlings of *Cicer arietinum* (21 g) were cut into small pieces and frozen with liquid nitrogen. The frozen material was powdered in Teflon tubes with a tungsten ball in a microdismembrator (Braun AG, Melsungen). The powder was added to 50 ml buffer C and 7 g Dowex 1X2. The slurry was stirred for 10 min and centrifuged at 23000 × g for 10 min. The supernatant was used as enzyme source.

2.9. *Other methods*

Protein was determined by a modified Lowry procedure [9] after precipitation of the protein with trichloroacetic acid in presence of deoxycholate [10].

3. RESULTS

3.1. *Product identification*

When the microsomal preparation from elicitor challenged soybean cell cultures [6,11] was incubated with (2*S*)-[^{14}C]naringenin (fig.1, I, R = OH) in presence of NADPH, a new radioactive product was formed which, on TLC with solvent 1, comigrated with genistein (fig.1, VI, R = OH). The identity of this product with genistein was unequivocally established by HPLC and reversed phase HPLC, by TLC with several solvent systems (see section 2) and by two-dimensional HPTLC with solvent 1 in the first and solvent 2 in the second direction. In each case the radioactive product coincided exactly with genistein. Furthermore, the product purified by HPLC was methylated in presence of *S*-adoMet with an enzyme from *Cicer arietinum* seedlings [12]. The

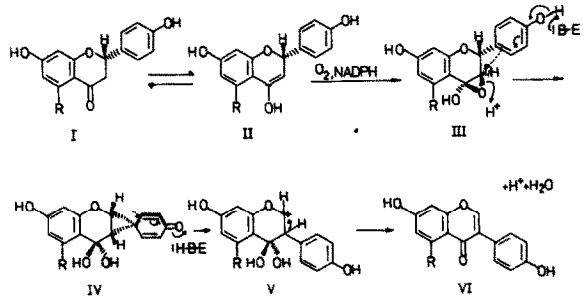

Fig.1. Hypothetical reaction sequence from (2*S*)-naringenin (I; R = OH) or (2*S*)-liquiritigenin (I, R = H) to genistein (VI, R = OH) or daidzein (VI, R = H).

reaction product was identified as 7-*O*-methylgenistein on TLC in solvents 2–5 by cochromatography with an authentic reference substance. Only in solvent 5 was separation of 7-*O*-methyl- and 4′-*O*-methylgenistein achieved. Identification was confirmed by two-dimensional HPTLC with solvents 2 and 5. Naringenin was not methylated by the enzyme preparation.

3.2. *Properties of isoflavone synthase*

The synthase showed an absolute requirement for reducing equivalents. As can be seen from table 1, NADH had only 4% of the relative activity of NADPH. With ascorbic acid, FMN, FAD or 6,7-dimethyl-5,6,7,8-tetrahydropterine alone no activity was detected. Simultaneous addition of NADPH and pteridine or $NADP^+$ caused inhibition of the reaction.

The oxygen dependence of the reaction was proved by incubation of the microsomes with an O_2-consuming system. Under these conditions only 3% of the original activity was observed (table 2). Addition of 10 μmol/l and 50 μmol/l cytochrome *c* to the synthase assay inhibited enzyme activity to 54 and 77%, respectively.

Besides (2*S*)-naringenin, (2*S*)-[5,4-^{14}C]dihydroxyflavanone (fig.1, I, R = H) was also a substrate for the synthase in the reaction leading to daidzein (fig.1, VI, R = H). The latter was identified by cochromatography with a reference sample in several solvents.

Table 1

Cofactor dependence of isoflavone synthase

Cofactor	Concentration	Activity (%)
None		0
NADPH	1 mmol/l	100
NADH	1 mmol/l	4
FAD	5 μmol/l	0
FMN	5 μmol/l	0
Ascorbic acid	1 mmol/l	0
NADPH + NADH	1 mmol/l + 1 mmol/l	103
NADPH + FAD	1 mmol/l + 5 μmol/l	95
+ FAD	1 mmol/l	0
NADPH + pteridin	1 mmol/l + 1 mmol/l	19
NADPH + NADH		62

Table 2

Isoflavone synthase after removal of oxygen with glucose oxidase

Addition	Activity (%)
None	100
Glucose 50 mmol/l	67
Glucose oxidase 5 U	59
Catalase 10 U	51
Glucose + glucose oxidase	4
Glucose + glucose oxidase + catalase	3

The incubation mixture without NADPH was incubated for 5 min in closed vials with the corresponding additions and then the reaction was started with NADPH. All values are average from two experiments

No synthase activity was detected in the supernatant of the 100000 × *g* centrifugation. The half-life at 30°C of synthase activity in buffer B was only about 10 min. At −70°C microsomes could be stored for several weeks without appreciable loss of activity.

3.3. *Elicitor-induction of synthase*

Six-day-old soybean cell cultures and 5-day-old soybean seedlings contained low but distinct synthase activity (21 and 16 μkat/kg, respectively). Challenge of the cell cultures for 18 h with the glucan elicitor from *P. megasperma* f.sp. *glycinea* caused an approximately 12-fold increase in enzyme activity. Elicitor induction at the hypocotyl of the seedlings caused an approximately 7-fold activity increase.

4. DISCUSSION

According to its cofactor requirements the microsomal isoflavone synthase is a monooxygenase. Inhibition by low concentrations of cytochrome *c* indicates that it could belong to the cytochrome *P*-450-dependent monooxygenases [13].

The microsomal preparation also contains chalcone isomerase (EC 5.5.1.6). Very probably, however, the flavanone is the substrate because the equilibrium of 4,2′,4′,6-tetrahydroxychalcone is

at least 1000:1 in favour of flavanone [14]. Furthermore, a stereospecific incorporation, in vivo, of (−)-(2*S*)-naringenin into biochanin A was found [15].

The hypothetical pathway from (−)-(2*S*)-flavanone to isoflavone shown in fig.1 is consistent with the participation of NADPH and dioxygen. Keto-enol tautomerism of flavanone is indicated by proton exchange at C-3 [16]. Epoxidation with cytochrome *P*-450 monooxygenases is known in higher plants [13].

The observation that isoflavone synthase activity is increased several-fold by treatment of soybean cell cultures or soybean seedlings with the glucan elicitor from *P. megasperma* f.sp. *glycinea* suggests that this enzyme plays a role in the regulation of phytoalexin (glyceollin) accumulation in this plant [17].

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