Isoflavone Synthase from Cell Suspension Cultures of *Pueraria lobata*[1)]

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Liquiritigenin and isoliquiritigenin were converted into daidzein by a microsomal fraction of cell suspension cultures of *Pueraria lobata* OHWI, which had been treated with an endogenous elicitor prepared by hydrolysis of their own cell walls with a fungal endopolygalacturonase. Competitive experiments with [^{3}H]flavanone and [^{14}C]chalcone as the substrates for isoflavone synthase revealed that flavanone is the true substrate for this enzyme reaction and the possible role of chalcone in the reaction is excluded.

Keywords *Pueraria lobata*; Leguminosae; kudzu; biosynthesis; flavonoid; isoflavone; daidzein; isoflavone synthase; cell suspension culture; endogenous elicitor

Pueraria lobata OHWI (Leguminosae) (Japanese name, "kudzu") has been known as the origin plant of "Puerariae Radix," an oriental medicinal drug used in Asian traditional medicines. The cell cultures induced from its root produce mainly 5-deoxy-type isoflavones,[2)] which are reputed to be the active principles of "Puerariae Radix."[3)] The isoflavonoid is a group of interesting natural products having various biological activities: oestrogenic, insecticidal, piscicidal and antifungal.[4)] Phytoalexins of leguminous plants are mostly isoflavonoids[5)] and the induction of isoflavonoid biosynthesis by various stimuli is an attractive topic of study.[6)]

The biosynthesis of flavonoid has been extensively studied at the enzyme level in many species of plants.[7)] The first enzyme in the pathway of common flavonoid biosynthesis is chalcone synthase, which catalyzes the formation of chalcone from *p*-coumaroyl Coenzyme A (CoA) and malonyl CoA. We recently reported the detection and characterization of deoxychalcone synthase in *P. lobata* cell cultures.[8)] Chalcone formed is further converted into a (−)-flavanone in a stereospecific manner by chalcone–flavanone isomerase.[9)] The reactions catalyzed by the two enzymes are recognized as the key steps in flavonoid biosynthesis. Limited information, however, is available on enzymes involved in the biosynthesis of isoflavonoids. Grisebach *et al.* reported the conversion of (2*S*)-naringenin (**2b**) into genistein (**3b**) (5-hydroxy-type isoflavone) by an enzyme preparation obtained from elicitor-challenged soybean cell cultures.[10)] Barz *et al.* reported on the series of enzymes related to isoflavonoid phytoalexins biosynthesis in *Cicer arietinum* cell suspension cultures.[11)]

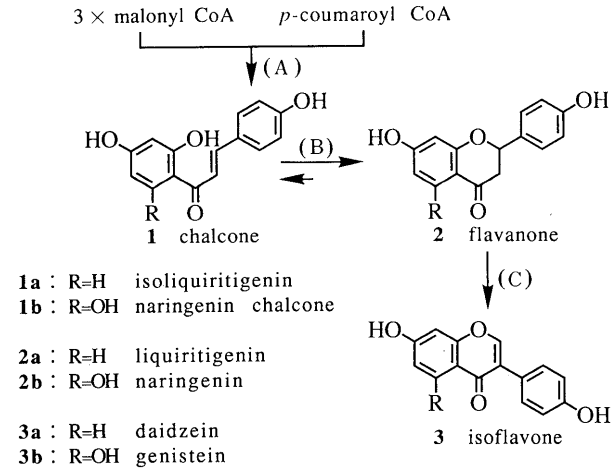

Fig. 1. Biosynthetic Pathway of Isoflavonoids in *Pueraria lobata*

The indicated enzyme reactions are catalyzed by (A) chalcone synthase, (B) chalcone–flavanone isomerase and (C) isoflavone synthase.

It has long been discussed whether flavanone or chalcone is the direct substrate in isoflavone formation. In soybean cell cultures, flavanone was assumed to be the substrate for isoflavone synthase, since the equilibrium of isomerase of this plant favors flavanone.[10b)] Although the selective incorporation of (2*S*)-naringenin over (2*R*)-isomer into isoflavone biochanin A was reported in *C. arietinum* seedlings,[12)] these results cannot exclude the possibility that chalcone is the substrate due to the presence of chalcone–flavonone isomerase which catalyzes the interconversion reaction between chalcone and flavanone. In order to clarify this question, we performed competitive experiments with [^{14}C]isoliquiritigenin (**1a**) and [^{3}H]liquiritigenin (**2a**) in the reaction of *P. lobata* isoflavone synthase, as we have briefly reported in an earlier communication.[1)] This paper describes full details of the experiments. The obtained results led to the conclusion that flavanone, but not chalcone, is the direct substrate of the isoflavone synthase.

Results

Detection of Isoflavone Synthase Activity When enzyme preparations from the normal cultured cells were tested for their isoflavone synthase activity using radioactive substrates, the enzyme activity was too low to give significant results. In order to overcome this problem, cellular fragments of *P. lobata* were chosen as an endogenous elicitor.[13)] The cultured cells challenged with this endogenous elicitor showed a reproducible high enzyme activity.

The microsomal preparation obtained from the cells treated with the endogenous elicitor was incubated with [^{14}C]isoliquiritigenin (**1a**) in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and the reaction products were separated and fractionated by reversed phase high performance liquid chromatography (HPLC). One of the radioactive products eluted with the same retention time as daidzein (**3a**). The fractions containing this product were pooled and applied on a silica gel thin layer chromatography (TLC). The radioactive product co-migrated with authentic daidzein (**3a**). Furthermore, the radioactive product which was purified with HPLC was diluted with non-labeled daidzein and recrystallized

TABLE I. Double Labeled Competitive Experiments with [^{3}H]Flavanone and [^{14}C]Chalcone for *P. lobata* Isoflavone Synthase

	Substrate		Recovered metabolite		
	Flavanone (**2a**)	Chalcone (**1a**)	Flavanone (**2a**)	Chalcone (**1a**)	Isoflavone (**3a**)
Experiment 1[a]					
^{3}H (dpm)	2.98×10^6 (2*S*)-**2a**	—	2.64×10^6 (2*S*)-**2a**		
^{3}H (dpm) (%)	1.49×10^6 (100)	—	1.15×10^6[c] (77.2)	1.78×10^5 (11.9)	3.20×10^4 (2.15)
^{14}C (dpm) (%)	—	1.97×10^4 (100)	1.24×10^4 (62.9)	3.07×10^3 (15.6)	8.70×10 (0.44)
(2*S*)-Fl/Ch[d]			1.23	0.76	4.89
Experiment 2[b]					
^{3}H (dpm) (%)	1.49×10^6 (100)	—	1.28×10^6 (85.9)	7.01×10^4 (4.70)	1.92×10^4 (1.29)
^{14}C (dpm) (%)	—	1.97×10^4 (100)	1.37×10^4 (69.5)	2.76×10^3 (14.0)	1.52×10^2 (0.77)
Fl/Ch[e]			1.24	0.34	1.68

a) Performed with 100 nmol racemic-[^{3}H]liquiritigenin (2.98×10^6 dpm) and 50 nmol [^{14}C]isoliquiritigenin (1.97×10^4 dpm), then original (2*R*)-liquiritigenin and (2*S*)-liquiritigenin was 50 nmol (1.49×10^6 dpm) each. *b*) Performed with 50 nmol racemic-[^{3}H]liquiritigenin (1.49×10^6 dpm) and 50 nmol [^{14}C]isoliquiritigenin (1.97×10^4 dpm). *c*) (dpm in flavanone fraction) − (dpm of original (2*R*)-flavanone). *d*) (incorporation (%) of (2*S*)-flavanone)/(incorporation (%) of chalcone). *e*) (incorporation (%) of racemic-flavanone)/(incorporation (%) of chalcone).

repeatedly to give a constant specific radioactivity (first: 8.51 dpm/mg, second: 8.97 dpm/mg and third: 8.54 dpm/mg). Thus the radioactive product was clearly identified as daidzein (**3a**).

Double Labeled Competitive Experiments All naturally occurring flavanones have (2*S*)-configuration. We carried out experiment 1 with [^{14}C]chalcone and racemic [^{3}H]-flavanone in a molar ratio of 1 : 2 (see Table I), by assuming that only (2*S*)-flavanone is the biosynthetically active isomer in *P. lobata*. A control experiment with boiled enzyme indicated that the non-enzymatic conversion between chalcone and flavanone was less than 1%. This excludes the involvement of non-enzymatic conversion in the experiments. As it appears in the (2*S*)-flavanone/chalcone ratio in Table I, the (2*S*)-flavanone pool was predominantly labeled by ^{3}H rather than ^{14}C which derived from [^{14}C]chalcone by the action of the isomerase. On the other hand, the chalcone pool was preferentially composed of original [^{14}C]chalcone. In this situation the ratio of (2*S*)-flavanone/chalcone in isoflavone formed was 4.89. The result clearly demonstrates that flavanone is the direct precursor of the rearrangement reaction yielding isoflavone.

When the incubation was performed with the same amount of [^{14}C]chalcone and racemic [^{3}H]flavanone (experiment 2), the flavanone/chalcone ratio in chalcone, flavanone and isoflavone was 0.34, 1.24 and 1.68, respectively. Even if isoflavone synthase of this plant has no stereoselectivity for substrate flavanone, the results obtained from experiment 2 led to the same conclusion mentioned above.[14]

Spectrometric Assay of Isoflavone Synthase In order to measure isoflavone synthase activity in a more conventional manner, a quantitative assay method was developed using non-labeled flavanone as substrate. The amount of isoflavone produced was calculated from the peak height in HPLC measured at 254 nm. Th reaction was linear up to 60 min and up to 250 μg with respect to protein. All the experiments discussed below were carried out by this assay method.

Properties of Isoflavone Synthase The isoflavone synthase required NADPH or reduced nicotinamide adenine dinucleotide (NADH) as a reducing cofactor. NADH showed only 30.8% of the NADPH activity at the same concentration. When the buffer B (see Experimetal) was degassed, the activity was markedly reduced (data not shown), suggesting that the enzyme requires molecular oxygen in the reaction. Microsomes could be stored at −80 °C in the presence of 14 mM 2-mercaptoethanol and 10% sucrose for at least 6 months without appreciable loss of activity. At 4 °C the half-life of enzyme activity was *ca.* 60 h.

Induction of Synthase Activity by Endogenous Elicitor Treatment When elicitor treated cells and control cells (treated with sterilized water) were harvested at the same time (20 h after the treatment), isoflavone synthase activities from equal weight of cells (fresh weight) were 1164 and 76 nkat/kg protein, respectively. Thus elicitor treatment caused more than 15-fold enhancement of enzyme activity.

Substrate Specificity Naringenin (**2b**: 5-hydroxy-flavanone) was also converted into the corresponding 5-hydroxy-isoflavone, genistein (**3b**), with the same enzyme preparation. From double-reciprocal plots, apparent K_m values for liquiritigenin (**2a**) and naringenin (**2b**) were calculated to be 6.9 and 20.0 μM, respectively. This suggests the isoflavone synthase in *P. lobata* cell suspension cultures favors 5-deoxy-flavanone rather than 5-hydroxy-flavanone.

Discussion

The role of chalcone–flavanone isomerase in isoflavonoid biosynthesis has remained unclear because decisive evidence on identification of the substrate of isoflavone synthase has not been available. Concomitant induction of this enzyme with phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) occurred by elicitor treatment, prior to the accumulation of isoflavonoid phytoalexin.[15] This suggests that chalcone–flavanone isomerase is one of the key enzymes in isoflavonoid biosynthesis.

The results so far obtained from the competitive experiments clearly demonstrate that flavanone, not chalcone, is the immediate precursor of isoflavone. This agrees with the conclusion obtained in a feeding experiment with *C. arietinum* seedlings.[12] The results obtained in this study have established chalcone–flavanone isomerase to be an essential enzyme for isoflavonoid biosynthesis, since it catalyzes the conversion of chalcone, the product of CHS, to yield flavanone, which is the substrate in the isoflavone

synthase reaction.

The cofactor requirement of the microsomal isoflavone synthase suggests the enzyme belongs to a class of monooxygenase; the postulated reaction mechanism reported by Grisebach[10a] is one possibility. An alternative mechanism was proposed by Crombie *et al.*[16] To clarify the true mechanism of this aryl migration, further studies using ^{18}O-labeled substrates or $^{18}O_2$ gas are now in progress.

P. lobata cell cultures produce mainly 5-deoxy isoflavones and 5-hydroxy isoflavones are minor constituents[2]; and endogeneous elicitor treatment of *P. lobata* cells causes the accumulation of deoxy-type isoflavonoid compounds such as daidzein. The isoflavone synthase of this plant is able to convert both 5-deoxy- and 5-hydroxy-type flavanones. K_m values, however, indicate that the isoflavone synthase has a preference for 5-deoxy-type flavanone. We previously paritially purified the chalcone–flavanone isomerase from *P. lobata* cell cultures and observed its substrate selectivity for isoliquiritigenin (**1a**) rather than naringenin chalcone (**1b**) (T. Hakamatsuka, unpublished data), and we recently reported the chalcone synthase activity which produced deoxychalcone in the presence of NADPH.[8] The induction of these three enzymes by endogenous elicitor treatment appears to be directly related to the accumulation of 5-deoxy-type isoflavonoids in *P. lobata* cell suspension cultures.

Experimental

Chemicals and Enzyme Daidzein, genistein and other flavonoids were from our collection. Endopolygalacturonase (*Aspergillus niger*) was obtained from Sigma. Other biochemicals were purchased from local suppliers and were of high quality.

Labeled Substrates [Carbonyl-^{14}C]isoliquiritigenin (3.94×10^{11} dpm/mol) was synthesized according to the reported method.[17] Racemic [^{3}H(G)]liquiritigenin (2.98×10^{13} dpm/mol) was prepared by the exchange reaction of chemically synthesized racemic liquiritigenin with ^3H_2O in the presence of triethylamine.[18]

***P. lobata* Cell Cultures** Callus cultures of *P. lobata* were derived from root as described.[2] Callus cultures were grown on Murashige–Skoog's agar medium supplemented with 2 ppm (2,4-dichlorophenoxy)acetic acid (2,4-D) and 0.1 ppm kinetin in 100 ml Erlenmeyer flasks at 25 °C in the dark. They were routinely subcultured every 20—25 d. Cell suspension cultures were initiated from callus cultures in Murashige–Skoog's medium and incubated at 25 °C in the dark in 500 ml Erlenmeyer flasks on a rotary shaker. One flask contained 100 ml of liquid medium. They were subcultured every 2 weeks.

Preparation of Endogenous Elicitor from *P. lobata* Cell Wall by Enzymic Digestion Six-day-old *P. lobata* suspension cultures were harvested and autoclaved. Fifty grams of the cells were suspended in 100 ml distilled water, homogenized first with a Waring blender and then with a sonicator. The homogenates were centrifuged at $2000 \times g$ for 5 min and the supernatant was discarded. The residue was resuspended in 100 ml distilled water. Washing by homogenization and centrifugation was repeated twice with distilled water and twice with Na-acetate (0.1 M, pH 5.2) buffer. The resultant residue was again suspended in 100 ml Na-acetate (0.1 M, pH 5.2) buffer, added with 600 units of endopolygalacturonase and incubated at 35 °C for 3 h with gentle shaking. The reaction was terminated by boiling for 10 min. The reaction mixture was centrifuged at $10000 \times g$ for 20 min and the supernatant was taken up and lyophilized. About 250 mg of amorphous powder was obtained and stored at room temperature.

For elicitor treatment, 5—7 mg of this powder was dissolved in 2 ml distilled water and added to 100 ml cell suspension cultures after autoclaving.

Chromatography Reversed phase HPLC was performed on a ODS-120A column (4.6 mm × 250 mm, Tosoh) with MeOH–H_2O gradient. The eluents were monitored at 254 nm and directly collected into scintillation vials using a fraction collector. The radioactivity was counted with a liquid scintillation counter.

TLC separation of reaction products was carried out with precoated silica gel TLC plate (Merck, 60F 254) using benzene–acetone (4 : 1) and $CHCl_3$–MeOH (9 : 1). Small sections were scrapped off from the plates into scintillation vials and counted for the radioactivity.

Buffer Buffer A: 0.1 M potassium phosphate, pH 7.5, containing 14 mM mercaptoethanol and 10% sucrose and degassed by an aspirator before use. Buffer B: same as buffer A but the degassing process was omitted.

Preparation of Microsomes All operations were carried out at 4 °C. *P. lobata* cells treated with endogenous elicitor for 20—24 h were harvested with a suction filter. The cells (50 g fresh weight) were frozen with liquid nitrogen and ground in a chilled mortar to a fine powder. The powder was added to 120 ml buffer A together with 5 g polyvinyl pyrolidone (PVP) and left for 10 min. The mixture was filtered through nylon cloth and the filtrate was stirred for 20 min with 10 g Dowex I × 2, pre-equilibrated with buffer A. After centrifugation at $20000 \times g$ for 30 min, the supernatant was further centrifuged at $105000 \times g$ for 60 min and the pellet was carefully washed twice with buffer B and taken up in 10 ml of this buffer. The slurry was homogenized in a glass homogenizer to give a microsome suspension.

Assay for Isoflavone Synthase The incubation mixture contained 1000 μl of microsomal fraction (1.5 mg protein) and 50 nmol of [carbonyl-^{14}C]isoliquiritigenin (1.97×10^4 dpm) and 50 nmol of NADPH in a total volume of 1040 μl. Incubation was carried out in a test tube for 40 min at 30 °C with shaking. The reaction was terminated by addition of 500 μl MeOH, and extracted with 2 ml EtOAc. The residue obtained by evaporation of EtOAc *in vacuo* was dissolved in 100 μl MeOH and applied to reversed phase HPLC. The eluent was fractionated and counted for the radioactivity as described above.

Double Labeled Experiments Experiment 1: The incubation mixture contained 1000 μl of microsomal fraction, 50 nmol of [carbonyl-^{14}C]isoliquiritigenin (1.97×10^4 dpm), 100 nmol of racemic [^{3}H(G)]liquiritigenin (2.98×10^6 dpm) and 50 nmol of NADPH in a total volume of 1060 μl. After incubation for 40 min at 30 °C, the reaction mixture was worked up and analyzed by HPLC as described above.

Experiment 2: The amount of racemic [^{3}H(G)]liquiritigenin was changed to 50 nmol (1.49×10^6 dpm) but other conditions were the same as experiment 1.

Spectrometric Assay Microsomal fractions were prepared as described above and diluted 5 times with buffer B. The incubation was carried out for 40 min at 30 °C with 500 μl of diluted microsomal fractions, 10 nmol of non-labelled racemic flavanone and 10 nmol of NADPH in a total volume of 540 μl. The reaction was stopped with 200 μl of MeOH and extracted twice with 1 ml EtOAc. The extract was evaporated and applied to reversed phase HPLC. The chromatogram was monitored at 254 nm. The amount of isoflavone produced was calculated from the peak height using a calibration curve obtained with standard samples.

References and Notes

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