ISOFLAVONE SYNTHASE FROM CELL SUSPENSION CULTURES OF PUERARIA LOBATA

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Isoflavone synthase activity was tested with a microsomal preparation of the cell cultures of Pueraria lobata that had been treated with an endogenous elicitor prepared by hydrolysis of their own cell walls with a fungal endopolygalacturonase. The deoxy types of flavanone and chalcone, liquiritigenin and isoliquiritigenin, were both converted into the corresponding isoflavone, daidzein, by a microsomal preparation. Competitive experiments with [^{3}H]flavanone and [^{14}C]chalcone revealed that flavanone is the direct substrate of this isoflavone synthase. Kinetic experiments indicated that liquiritigenin is a much more favorable substrate than naringenin.

KEYWORDS —— isoflavone synthase; Pueraria lobata; daidzein; isoflavonoid; biosynthesis; endogenous elicitor

Pueraria lobata Ohwi (Leguminosae) (Japanese name kudzu) is source of Puerariae Radix, which has been used in Asian traditional medicine for a long time. The main secondary metabolites produced by cell cultures of P. lobata are daidzin and puerarin, 5-deoxy-type isoflavone glycosides.[1] They were considered to be active principles in traditional medicines.[2]

The biosynthesis of flavonoid has been extensively studied at the enzyme levels in many species of plants,[3] since flavonoid biosynthesis is one of the major pathways of secondary metabolism in the plant kingdom. The entrance enzyme of flavonoid biosythesis is chalcone synthase which catalyzes the formation of chalcone from p-coumaroyl and malonyl CoAs. In a previous paper we reported the detection and characterization of deoxychalcone synthase in P. lobata cell cultures.[4] Chalcone is further converted into a (-)-flavanone in a stereospecific manner by chalcone-flavanone isomerase.[5] The reactions catalyzed by these two enzymes are recognized as the key steps in flavonoid biosynthesis that branch from the general pathway of phenylpropanoid biosynthesis. Limited information is available on the biosynthesis of 5-deoxy-type isoflavonoids, which are the common secondary metabolites of legumes.[6,7] Isoflavone synthase is the critical enzyme of isofalvonoid biosynthesis and catalyzes B-ring migration in the C_6-C_3-C_6 skeleton. Grisebach et al. reported the conversion of naringenin into genistein by an enzyme preparation obtained from soybean cell cultures treated with a fungal elicitor.[8] However, their experimental data reported for the 5-hydroxy-type isoflavone formation cannot completely exclude the possibility that chalcone is

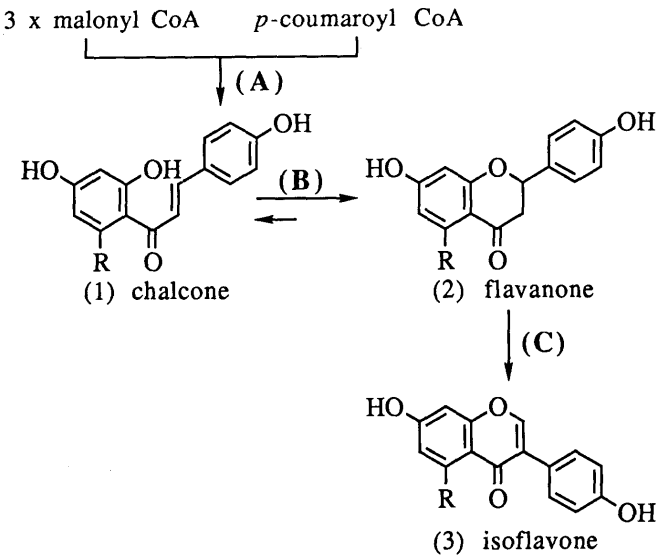

Chart 1. Biosynthetic Pathway of Isoflavonoids in *P. lobata*

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

(1a) R=H isoliquiritigenin
(1b) R=OH naringenin chalcone

(2a) R=H liquiritigenin
(2b) R=OH naringenin

(3a) R=H daidzein
(3b) R=OH genistein

the direct substrate of isoflavone synthase,[8,9,10] since chalcone-flavanone isomerase inevitably contaminates the enzyme preparations and catalyzes the reverse conversion of flavanone into corresponding chalcone.

We have studied the enzymic conversion of chalcone and flavanone into isoflavone using microsomal preparations of P. lobata cultured cells. When microsomal fractions were prepared from normal cultured cells, the formation of isoflavones from radioactive chalcones and falvanones was not detected. An endogenous elicitor[11] was prepared as described in a previous paper[4] and elicitor treatment of the cultures resulted in successful detection of isoflavone synthase activity. Radioactive daidzein formed from [^{14}C]isoliquiritigenin was separated and isolated by HPLC. The radioactive sample was diluted with non-labeled daidzein and recrystallized three times. The samples showed constant specific activity (first; 8.51 dpm/mg, second; 8.97 dpm/mg and third; 8.54 dpm/mg) and the formation of daidzein from isoliquiritigenin was rigorously established. The reaction requires NADPH as a cofactor. NADH was only ca. 30% as active as NADPH. The reaction rate was greatly decreased, when incubation was carried out with a degassed buffer, indicating that oxygen is required in the raction.

To identify the direct substrate of isoflavone synthase, competitive experiments were carried out using [^{14}C]isoliquiritigenin (chalcone) and [^{3}H]liquiritigenin (flavanone). After incubation, recovered substrates and the product were separated by HPLC and their $^{3}H/^{14}C$ ratios were measured to determine the degree of contribution in the reaction (See Table 1). The $^{3}H/^{14}C$ ratio of chalcone and flavanone were 0.77 and 1.23, respectively. In the incubation periods, the isoliquiritigenin pool was more highly labeled by ^{14}C, but ^{3}H was dominant in the liquiritigenin pool. Under the reaction conditions, the $^{3}H/^{14}C$ ratio of daidzein was 4.89. This clearly indicated that liquiritigenin is the direct substrate of isoflavone synthase, since it is highly unlikely that both chalcone and flavanone serve as the direct substrates of isoflavone synthase.

Table 1. Double Labeled Competitive Experiments with [^{3}H]Flavanone and [^{14}C]Chalcone for P. lobata Isoflavone Synthase

	Substrates[a]		Recovered metabolites		
	Flavanone	Chalcone	Flavanone	Chalcone	Isoflavone
^{3}H (dpm)	2.98×10^6	—	2.64×10^6		
	(2S)-Flavanone		(2S)-Flavanone[b]		
^{3}H (dpm)	1.49×10^6	—	1.15×10^6	1.78×10^5	3.20×10^4
(%)	(100)		(77.5)	(12.0)	(2.15)
^{14}C (dpm)	—	1.97×10^4	1.24×10^4	3.07×10^3	
(%)		(100)	(63.0)	(15.6)	(0.44)
^{3}H/^{14}C ratio[c]			1.23	0.77	4.89

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). The original (2R)- and (2S)-[^{3}H]liquiritigenin were 50 nmol (1.49×10^6 dpm) each.

b) (dpm in flavanone fraction) - (dpm of original (2R)-[^{3}H]flavanone).

c) (incorporation (%) of (2S)-[^{3}H]flavanone) / (incorporation (%) of [^{14}C]chalcone).

To compare the efficiency of 5-hydroxy-type and 5-deoxy-type flavanone, naringenin and liquiritigenin, in the reaction of isoflavone synthase, kinetic experiments were carried out to determine the Km values for the substrates. The apparent Km values for naringenin and liquiritigenin were calculated to be 20.0 μM and 6.9 μM, respectively. These results indicate that the isoflavone synthase in P. lobata showed specificity for 5-deoxy-type flavanone.

Endogenous elicitor treatment of P. lobata cells causes the accumulation of 5-deoxy-type isoflavonoid compounds such as daidzein.[12] The activity of chalcone synthase, chalcone-flavanone isomerase and isoflavone synthase, the three enzymes involved in isoflavonoid biosynthesis, were detected in P. lobata cell suspension cultures treated with the endogenous elicitor. Chalcone synthase of P. lobata produced deoxychalcone in the presence of NADPH.[4] Chalcone-flavanone isomerase, as well as isoflavone synthase, showed specificity for deoxy-type substrates.[12] The induction of the three enzymes above by endogenous elicitor treatment appears to be directly related to the accumulation of 5-deoxy-type isoflavonoids in P. lobata cell suspension cultures.

MATERIALS AND METHODS

Materials [carbonyl-^{14}C]Isoliquiritigenin (3.94×10^8 dpm/mmol) was synthesized according to a reported method.[13] Racemic-[^{3}H(G)]liquiritigenin (2.98×10^{10} dpm/mmol) was prepared by exchanging reaction of racemic-liquiritigenin with ^3H_2O in the presence of triethylamine.[14]

Cell Cultures Cell cultures of P. lobata were established and challenged

with the elicitor as described in a previous communication.[4)]

Enzyme Preparation The elicitor-challenged cells (50 g fresh weight) were frozen with liquid nitrogen and ground in a chilled mortar to a fine powder. This powder was added to degassed buffer (120 ml, pH 7.5) consisting of potassium phosphate (0.1 M), 2-mercaptoethanol (14 mM) and sucrose (10%) together with polyvinylpyrrolidone (5 g), and left to stand for 10 min. The mixture was filtered through nylon gauze and the filtrate was stirred for 20 min with 10 g Dowex I X 2, pre-equilibrated with the same buffer. After centrifugation at 20,000 x g for 30 min, the supernatant was further centrifuged at 105,000 x g for 60 min and the pellet was carefully washed twice with non-degassed buffer and taken up in 10 ml of non-degassed buffer. The slurry was homogenized in a glass homogenizer to give the microsomal preparation used for the isoflavone synthase experiments.

Assay of Isoflavone Synthase The incubation mixture contained 1 ml 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 1.04 ml. Incubation was carried out in a test tube for 40 min at 30°C with shaking. The reaction was terminated by adding 0.5 ml MeOH, and product was extracted with 2 ml EtOAc. The residue obtained after removing the solvent was dissolved in 0.1 ml MeOH and submitted to reversed phase HPLC with ODS-120T column (4.6 mm x 250 mm, Tosoh) and a UV monitor (254 nm). The column was eluted with MeOH-H_2O (40-80% gradient) and fractions corresponding to the peak of daidzein were collected for radioactive measurement (Aloka Scintillation Counter). The assay for the kinetic studies was carried out, using non-labeled flavanones as substrates, by direct measurment of the product formation based on the peak height in HPLC analysis (UV detection at 254 nm).

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