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DEOXYCHALCONE SYNTHASE FROM CELL SUSPENSION CULTURES OF PUERARIA LOBATA

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Chalcone synthase of Pueraria lobata cell cultures treated with an endogenous elicitor afforded isoliquiritigenin, a deoxy-type chalcone, from p-coumaroyl and malonyl CoAs in the presence of NADPH. But naringenin chalcone, a hydroxy-type chalcone, was the sole reaction product when NADPH was omitted from the reaction mixture. At lower NADPH concentrations, the formation of isoliquiritigenin was proportional to the concentration of NADPH and reached its maximum at ca. 1 mM. The total amount of the chalcones formed was constant throughout the range of 0 to 4 mM NADPH. These results seem to indicate that a single deoxychalcone synthase (DOCS) gives either isoliquiritigenin or naringenin chalcone depending upon the presence or absence of NADPH.

KEYWORDS ——— chalcone synthase; Pueraria lobata; Leguminosae; biosynthesis; isoflavonoid; deoxychalcone; endogenous elicitor

Pueraria lobata Ohwi (Leguminosae) (Japanese name kudzu) is an original plant of Puerariae Radix, which has been used as an oriental medicinal drug in Asian traditional medicines. The cell cultures of P. lobata produce 5-deoxy-type isoflavonoids,[1] daidzin and puerarin, which are reputed to be the active principles of Puerariae Radix. Since flavonoid is the most frequently found plant constituent, the biosynthesis of flavonoid has been extensively studied.[2] The first enzyme in the pathway of flavonoid biosynthesis is chalcone synthase, which catalyzes the formation of chalcone from p-coumaroyl and malonyl CoAs. The chalcone thus formed is further converted into flavanone by the second enzyme, chalcone-flavanone isomerase. The reactions catalyzed by the two enzymes are recognized as the general steps of flavonoid biosynthesis. The presence of chalcone synthase was first demonstrated in cell suspension cultures of parsley in which flavonoid biosynthesis had been induced by light.[3] This was followed by the extensive investigations in soybean,[4] Phaseolus vulgaris,[5] tulip,[6] carrot,[7] etc. All of the chalcone synthases reported to date have been hydroxy-type chalcone synthases (CHSs) which yielded naringenin chalcone (2',4,4',6'-tetrahydroxychalcone). But limited information is available concerning the biosynthetic enzymes of 5-deoxy-type isoflavonoids, which are the common secondary metabolites of leguminous plants.[8] Recently, Ayabe et al. succeeded in detecting the activity of deoxy-type chalcone synthase (DOCS) in tissue cultures of Glycyrrhiza echinata,[9] which produced deoxy-type flavonoids as the main

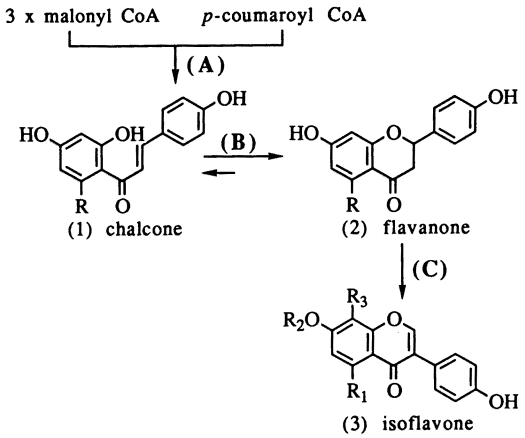

Chart 1. Biosynthetic Pathway of Isoflavonoids in *P. lobata*

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

(3c) R_1=H, R_2=Glc, R_3=H daidzin
(3d) R_1=H, R_2=H, R_3=Glc puerarin

constituents. This prompted us to report our results from enzymatic studies of isoflavonoid biosynthesis in P. lobata cell cultures.

Since the loss of the oxygen atom at C-5 in isofalvonoids occurs prior to the aromatization of the chalcone skeleton,[10] DOCS which affords isoliquiritigenin (2',4,4'-trihydroxychalcone), should play a main role in isoflavonoid biosynthesis. To obtain highly active enzyme preparations, the cell cultures of P. lobata were tested with different types of elicitors. Of those tested, the best results, including activity and reproducibility, were obtained with an endogenous elicitor[11] which was prepared by the hydrolysis of P. lobata cell wall fraction with a fungal endopolygalacturonase. When chalcone synthase was assayed with a soluble enzyme preparation using p-coumaroyl and [^{14}C] malonyl CoAs as substrates in the absence of NADPH, the radioactivity of the reaction products fell in the HPLC fractions of naringenin, which had been derived from naringenin chalcone by chalcone-flavanone isomerase. But two additional radioactive products were formed in the presence of NADPH. They have the same retention times as isoliquiritigenin and liquiritigenin, and their identity has been established by dilution analysis with authentic samples. The result indicates that in the presence of NADPH the cell-free preparation does contain the enzyme forming isoliquiritigenin from p-coumaroyl and malonyl CoAs, and that the isoliquiritigenin formed was further converted into liquiritigenin by the chalcone-flavanone isomerase present in the enzyme preparation. As shown in Fig. 1, the sum of isoliquiritigenin and liquiritigenin formed in the reaction is proportional to the concentration of NADPH in the range of 0 to 1 mM, and it stayed at the same level up to 4 mM. The total amount of chalcone, which is caluculated as the sum of the three reaction products, stayed more or less at the same level throughout the range of 0 to 4 mM NADPH. The ratio of isoliquiritigenin formation to total chalcone formation was ca. 45% at the maximum point. The ratio varied in each experiment with enzyme preparation, pH, and ionic strength, indicating that the reaction conditions affected the ratio of isoliquiritigenin formation.

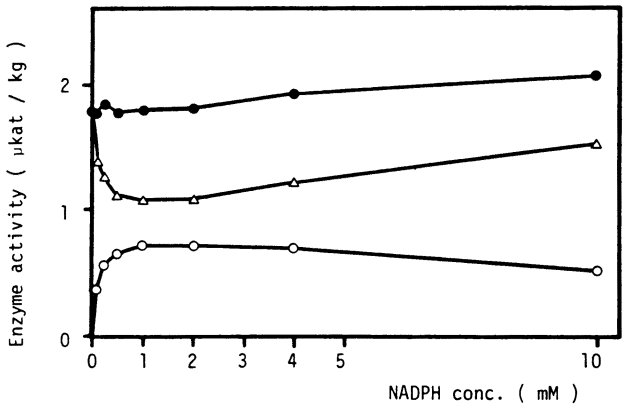

Fig. 1. Dependence of Chalcone Synthase Activity on NADPH Concentration

● total chalcone formation [isoliquiritigenin (I) + liquiritigenin (L) + naringenin (N)];

○ 5-deoxy chalcone formation [I + L];

△ 5-hydroxy chalcone formation [N].

(Numbering is based on flavonoid skeleton.)

Although several interpretations are possible to account for the experimental results, we prefer the one that is based on the presence of a single DOCS consisting of two distinct moieties. A moiety responsible for the loss of oxygen function is assumed to be rather unstable and is partially inactivated during enzyme preparation, while the other moiety, corresponding to the template of polyketide synthesis, is kept intact. As a result, the enzyme yielded both naringenin chalcone and isoliquiritigenin when NADPH was present, whereas only naringenin chalcone was formed when NADPH was absent. Since fully active DOCS can afford the same amount of naringenin chalcone instead of isoliquiritigenin in the absence of NADPH, the total amount of chalcone formed by the whole enzyme is constant. If we assume the presence of two different enzymes, CHS and DOCS, the role of CHS in P. lobata is difficult to understand, because the production of 5-hydroxy-type isoflavonoids in P. lobata is very limited.[1] At this moment, it is not clear whether DOCS is a single protein or a multi-enzyme complex. The purification of DOCS is in progress in our laboratory.

Two different mechanisms have been known for the deoxygenation of acetate-derived oxygen in polyketide biosynthesis. Recently, an enzyme to yield chrysophanol from emodin, an aromatic anthraquinone, was reported and the involvement of a reduction step by NADPH was rigorously established by NMR studies.[13] In the biosynthesis of 6-methylsalicylic acid,[12] the loss of oxygen atom occurs before aromatization, as in DOCS reaction. If NADPH was omitted from the reaction mixture, triacetic acid lactone was formed as an intermdiate in an incomplete biosynthetic reaction. This gives quite a contrast to DOCS. DOCS is a new type of polyketide synthetic enzyme which affords a deoxy-type compound in the presence of NADPH and a hydroxy-type compound in the absence of NADPH.

EXPERIMENTAL

Cell Cultures Cell suspension cultures of P. lobata were established from callus cultures which had been induced from its root as reported.[1] The

cultures were maintained in Murashige-Skoog's medium containing 2,4-D (2 ppm), kinetin (0.1 ppm) and sucrose (3%).

Preparation of Endogenous Elicitor and Treatment of Cell Cultures The cells of 6-day-old P. lobata suspension cultures were used for cell wall hydrolysis. It was prepared according to a known procedure using the endopolygalacturonase of Aspergillus niger (SIGMA).[14] Sterilized endogenous elicitor (5-7 mg) was added to 100 ml of cell suspension cultures (7 days old) and incubated for 18-22 h. The cells were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until use.

Enzyme preparation and Assay The P. lobata cells (5 g) were ground in a mortar for 5 min together with sand (3 g) and buffer solution (8 ml, pH 6.0) consisting of potassium phosphate (0.1 M), ascorbate (20 mM) and glycerol (10%). The homogenate was centrifuged at 20,000 x g and the supernatant was treated with Dowex I x 2. The mixture was filtered through sintered glass and centrifuged at 105,000 x g for 60 min. The supernatant of ultracentrifugation was used as a soluble enzyme in chalcone synthase experiments. The assay mixture (total volume 100 μl) contained [^{14}C]malonyl CoA (20 nmol, 49.5 mCi/mmol), p-coumaroyl CoA (25 nmol),[15] NADPH (200 nmol) and 70 μl enzyme preparation (100-150 mg protein). After incubation for 30 min at 30°C, the reaction products were extracted and submitted to HPLC separation with an ODS-120T column (4.6 mm x 250 mm, Tosoh). The column was eluted with MeOH-H_2O (40-80% gradient) and monitored with a UV detector (254 nm). Fractions corresponding to the peaks of products were directly collected into scintillation vials for radioactive measurement.

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