

ENZYMATIC SYNTHESIS OF AROMATIC COMPOUNDS IN HIGHER PLANTS: FORMATION OF NARINGENIN (5, 7, 4'-TRIHYDROXYFLAVANONE) FROM p-COUMAROYL COENZYME A AND MALONYL COENZYME A

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1. Introduction

As an extension of Birch's hypothesis [1] Grisebach postulated that in analogy to fatty acid synthesis flavonoid compounds are formed from CoA thiol esters of cinnamic acids and malonate [2, 3]. An intermediate enzyme-bound β -triketo acid was suggested to be the immediate precursor of a chalcone which can be further cyclized by a chalcone-flavanone isomerase to give the corresponding flavanone (fig. 1).

Recently, most of the enzymes related to the biosynthesis of flavone glycosides were demonstrated in cell suspension cultures from parsley (*Petroselinum hortense*) after treatment of the cells with light [4]. Based upon highly coordinated changes in the activities of these enzymes it was postulated that a thus far hypothetical "chalcone synthetase" is most active about 24 hr after the onset of illumination.

In this communication, we report for the first time the cell-free formation of a flavonoid (5, 7, 4'-trihydroxyflavanone) from p-coumaroyl CoA and malonyl CoA by an enzyme preparation from illuminated parsley cell suspension cultures. Evidence is presented that the aromatic "ring A" of the flavanone is derived from malonate while "ring B" originates from the phenyl ring of p-coumarate (cf. fig. 1).

2. Experimental

2.1. Methods

Cell suspension cultures of parsley (*Petroselinum hortense*) were grown, illuminated for 24 hr, and harvested as described previously [4].

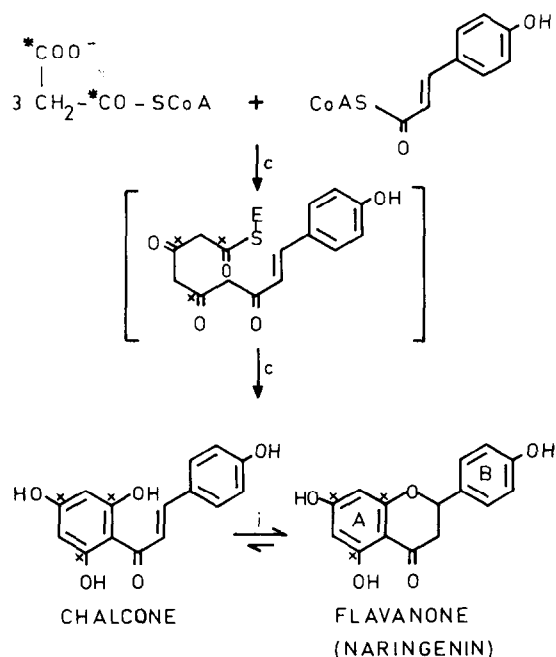


Fig. 1. Hypothetic scheme for the enzymatic synthesis of a flavanone from malonyl CoA and p-coumaroyl CoA: c = reactions catalysed by a "chalcone synthetase"; i = reaction catalysed by a chalcone-flavone isomerase; E = enzyme.

A crude enzyme preparation was obtained by homogenizing 5 g (fresh weight) of 11-day-old cells for 3 min in a chilled mortar with 2.5 g of quartz sand and 2.5 ml of 0.02 M potassium phosphate buffer (pH 7.5) containing 1 mM mercaptoethanol. After centrifugation for 15 min at 15,000 g, the supernatant was stirred for 20 min at 4° with 0.5 g of Dowex

Table 1
Variations of the standard incubation mixture

Radioactive substrate	Assay conditions	Radioactivity incorporated into naringenin (dpm)	Product formed* (nmoles)
[1, 3- ¹⁴ C] Malonyl CoA	Complete	50,000	0.8
[1, 3- ¹⁴ C] Malonyl CoA	– p-Coumaroyl CoA	Not detectable	–
[1, 3- ¹⁴ C] Malonyl CoA	Protein extract from non-illuminated cells	Not detectable	–
[1, 3- ¹⁴ C] Malonyl CoA	Boiled enzyme	Not detectable	–
[2- ¹⁴ C] p-Coumaroyl CoA	Complete**	3,400	1

* Calculated from the amount of radioactivity incorporated into naringenin and corrected for the loss of one-half of the label when [1, 3-¹⁴C] malonyl CoA was used as a precursor.

** The incubation mixture contained 120 nmoles of [2-¹⁴C] p-coumaroyl CoA (400,000 dpm) and 100 n moles of unlabelled malonyl CoA.

I X 2 (phosphate form, equilibrated with 0.2 M potassium phosphate, pH 7.5), and the resin then removed by filtration through glass wool. The filtrate was used directly for studies on the enzymatic synthesis of the flavanone.

Incubation of 200 μ l of the enzyme preparation and 5 nmoles of [1,3-¹⁴C] malonyl CoA (220,000 dpm) with a mixture containing 50 nmoles of p-coumaroyl CoA, 5 μ moles of ATP, and 20 μ moles of potassium phosphate (pH 7.5) in another 200 μ l was carried out at 30° for 120 min. The reaction was stopped by adding 30 μ g of naringenin dissolved in 30 μ l of methanol, and evaporating the mixture on chromatography paper (Schleicher-Schuell, No. 2043 b). Labelled naringenin was identified by chromatog-

raphy in five different solvent systems (20% ethanol; 15% acetic acid; 30% acetic acid; tert.-butanol/acetic acid/H₂O, 3:1:1; benzene/acetic acid/H₂O, 115:72:3). For standard assays (see table 1), the chromatogram was developed in 20% ethanol. Radioactive peaks corresponding to the flavanone were measured by scintillation spectrometry.

2.2. Materials

Non-radioactive and 2-¹⁴C-labelled (1.5 mCi/mmole) p-coumaroyl CoA was synthesized enzymatically as described elsewhere [5]. [1,3-¹⁴C] Malonyl CoA (18.5 mCi/mmole) was purchased from New England Nuclear, Boston, Mass. Unlabelled malonyl CoA was from Serva, Heidelberg.

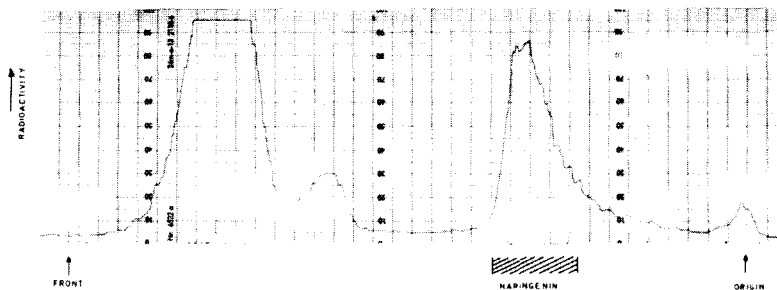


Fig. 2. Identification of naringenin by paper chromatography of the incubation mixture in 20% ethanol. Substrates were p-coumaroyl CoA and 1, 3-¹⁴C-labelled malonyl CoA as described under Methods. Radioactivity was recorded in a paper chromatogram scanner LB 280 (Berthold, Wildbad).

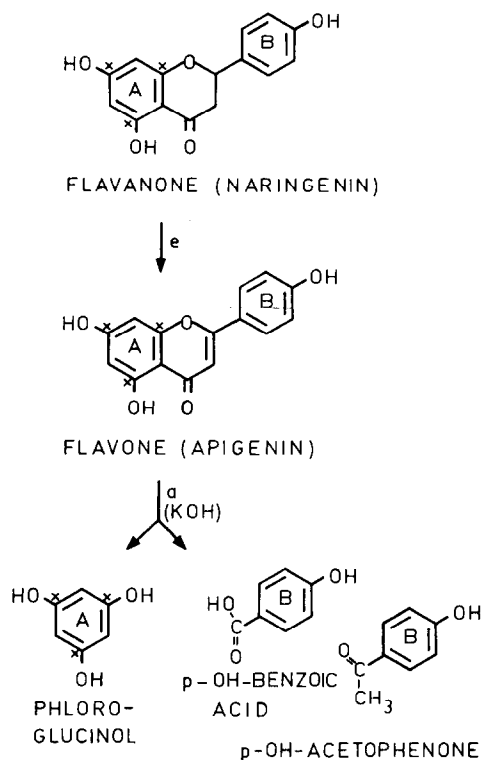


Fig. 3. Determination of radioactivity in ring A and ring B of enzymatically synthesized naringenin. e = Enzymatic oxidation of a flavanone to a flavone with a crude protein extract from leaves of young parsley plants [6]. The extract was obtained by homogenizing 2 g of plant material at 4° in a mortar with 2 g of quartz sand and 3 ml of Tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol. Insoluble material was removed by centrifugation (15 min at 15,000 g). 2 ml of the supernatant were incubated with 0.1 μ mole of naringenin (550,000 dpm; dissolved in 50 μ l of ethylenglycol monomethyl ether) at 30° for 3 hr. The reaction was stopped by adding 20 μ l of formic acid and extracting the aqueous solution with diethyl ether (7 \times 5 ml). The combined ether fractions were evaporated, and the residue was chromatographed on Schleicher-Schuell No. 2043 b paper in 30% acetic acid. The radioactive peak corresponding to the flavone was cut out and eluted with methanol. a = Alkaline cleavage of the isolated apigenin (150,000 dpm) after dilution with 1 mg of unlabelled material [7]. The products were identified by paper chromatography (benzene/acetic acid/H₂O, 6:7:3) and TLC (chloroform/acetic acid/H₂O, 50:45:5) on cellulose.

3. Results and discussion

After incubation of a protein extract from illuminated parsley cell cultures with [1, 3-¹⁴C] malonyl CoA

and p-coumaroyl CoA, a radioactive product was isolated by paper chromatography (fig. 2) which was identical with an authentic sample of naringenin in five different solvent systems.

The flavanone was further characterized by its enzymatic conversion to the corresponding flavone (as in fig. 3). The radioactivity of the isolated product coincided with the *R_f* values of apigenin in four different solvent systems. In order to determine the position of the labelled carbon atoms, the radioactive material was diluted with carrier flavone and subjected to alkaline cleavage of the heterocyclic ring (fig. 3). By this method, ring A of the flavone is converted to phloroglucinol while ring B gives rise to a mixture of p-hydroxybenzoic acid and p-hydroxyacetophenone [7]. When the products of this reaction were isolated and chromatographed in two different solvent systems, radioactive label was solely associated with the spots corresponding to phloroglucinol and non-converted apigenin. Both p-hydroxybenzoic acid and p-hydroxyacetophenone were unlabelled. This demonstrates that the radioactivity of [1, 3-¹⁴C] malonyl CoA was incorporated into ring A of naringenin, while ring B was not labelled. These results are in agreement with the hypothetic scheme shown in fig. 1.

Additional evidence for the specific incorporation of malonyl CoA and p-coumaroyl CoA into ring A and ring B, respectively, is presented in table 1. Comparable amounts of radioactive naringenin were formed when either precursor was ¹⁴C-labelled. No naringenin was detected when p-coumaroyl CoA was omitted from the assay mixture, when the enzyme was heat-inactivated, or when an extract from non-illuminated cells was used. The latter observation suggests that this enzyme is induced by light and could be regulated after illumination as postulated in a previous publication [4].

It should be mentioned that ATP was not required for the condensation reaction. However, a slight increase in the formation of naringenin was observed when ATP was added to the incubation mixture, probably due to the presence of CoA ligases in the protein extract [8] which could reactivate free acids formed by hydrolysis of malonyl and coumaroyl CoA.

We have also been able to demonstrate the formation of naringenin with cell-free preparations from very young parsley leaves harvested from 21-day-old

plants. By contrast, no flavanone was formed with extracts from leaves of 30-day-old parsley plants. This difference in activity is in agreement with earlier observations on changes of the activities of other enzymes related to flavonoid biosynthesis in this plant [9].

Acknowledgements

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