ENZYMATIC SYNTHESIS OF CHLOROGENIC ACID FROM CAFFEOYL COENZYME A AND QUINIC ACID

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1. Introduction

Chlorogenic acid (3-*O*-caffeoyl-D-quinic acid) and a variety of closely related hydroxy cinnamoyl conjugates of both quinic acid and shikimic acid are of widespread occurrence in plant tissues [1 and literature cited therein]. Caffeoylquinic acid is assumed to be formed by esterification of quinic acid with cinnamic acid, unidentified carbohydrate esters of cinnamic acid being suspected as intermediates [2], and subsequent hydroxylation of the cinnamic acid moiety first in the 4, then in the 3 position [3–5]. In contrast, Gamborg [6] using potato cell suspension cultures demonstrated clearly that both quinic acid and caffeic acid serve as direct precursors of chlorogenic acid in these cells.

Esterification of quinic acid with a cinnamoyl or hydroxy cinnamoyl moiety represents an endergonic process, which most likely requires activation of the carboxyl group of the phenylpropane derivative. It has been suggested that the actual pool of 'activated' phenylpropanoid intermediates involved in the biosynthesis of lignin, flavonoids, chlorogenic acid, etc., is to be found in acetone insoluble protein-bound cinnamic acid derivatives [7,8]. On the other hand, it has been postulated that the energy-rich intermediates in these reactions are low molecular weight hydroxy cinnamoyl-CoA derivatives [9 and literature cited therein]. This latter assumption has been proven to be correct for the biosynthesis of a flavonoid [10] and of coniferyl alcohol, the main lignin precursor [11–13]. It appeared therefore under this aspect to be appropriate to reinvestigate the enzymatic synthesis of chlorogenic acid.

In this communication we report that cell free preparations of cell suspension cultures of *Nicotiana alata* activate caffeic acid in the presence of ATP and CoA to caffeoyl-CoA and transfer the caffeoyl moiety of this thiol ester to quinic acid to form chlorogenic acid.

2. Experimental

2.1. Methods

Cell suspension cultures of *Nicotiana alata* were grown in the light at 23°C in B 5 medium [14] supplemented with caseinehydrolysate, indole-3-acetic acid, 1-naphthalene acetic acid and kinetin as well as 5 g per liter quinic acid at pH 5.5 for 11 days. Tissue was harvested by filtration, immediately frozen with liquid nitrogen and stored at −20°C.

A crude enzyme preparation was obtained by crushing the frozen cells in a BIO X-press and subsequent treatment of the extract as described previously [12]. Hydroxy cinnamate: CoA ligase was assayed by an optical method [15]. Incubation of the enzyme preparation with various cofactors and substrate was carried out at 30°C for 180 min. The reaction was stopped by adding unlabelled carrier chlorogenic acid in ethanol and evaporation of an aliquot of the incubation mixture on chromatography paper (Whatman No. 3). The chromatogram was developed ascending using *n*-butanol: glacial acetic acid: H_2O = 4:1:5 (organic phase) as solvent system. Labelled chlorogenic acid was identified as pentaacetyl-

chlorogenic acid, which was recrystallized from four different solvents to constant specific activity, as well as by paper chromatography in six different solvent systems (10 % acetic acid; *n*-butanol: 2 N HCl = 4:6 (organic phase): methylisobutylketone: HCO_2H : H_2O = 14:3:2; *n*-butanol : pyridine : H_2O = 14:3:3; isopropanol:H_2O = 7:3; ethyl acetate: HCO_2H:H_2O = 15:1:5).

2.2. *Material*

Trans-[2-^{14}C]caffeic acid was synthesized from protocatechu aldehyde and [2-^{14}C]malonic acid according to standard procedures. [G-^{14}C]quinic acid was a gift of Dr. E. Beck, Munich. Caffeoyl-CoA was synthesized chemically as described elsewhere [16]. Mass spectra were recorded on a Varian MAT 111.

3. Results and discussion

Cell cultures of seven different *Nicotiana* species were analyzed for their content of chlorogenic acid. It was found that *N. alata* contained an appreciable amount of this depside, which was increased considerably by growing the cells in a medium containing quinic acid. After incubation of a protein fraction (35–72% ammonium sulfate) from these cells with caffeic acid, quinic acid (either one labelled with ^{14}C), ATP, CoA, Mg^{2+} and a reduced thiol, in both cases a labelled product was isolated by paper chromatography (fig. 1) which was identified as chlorogenic acid in the following way. The ^{14}C-labelled product from an experiment using [2-^{14}C] caffeic acid was diluted with unlabelled chlorogenic

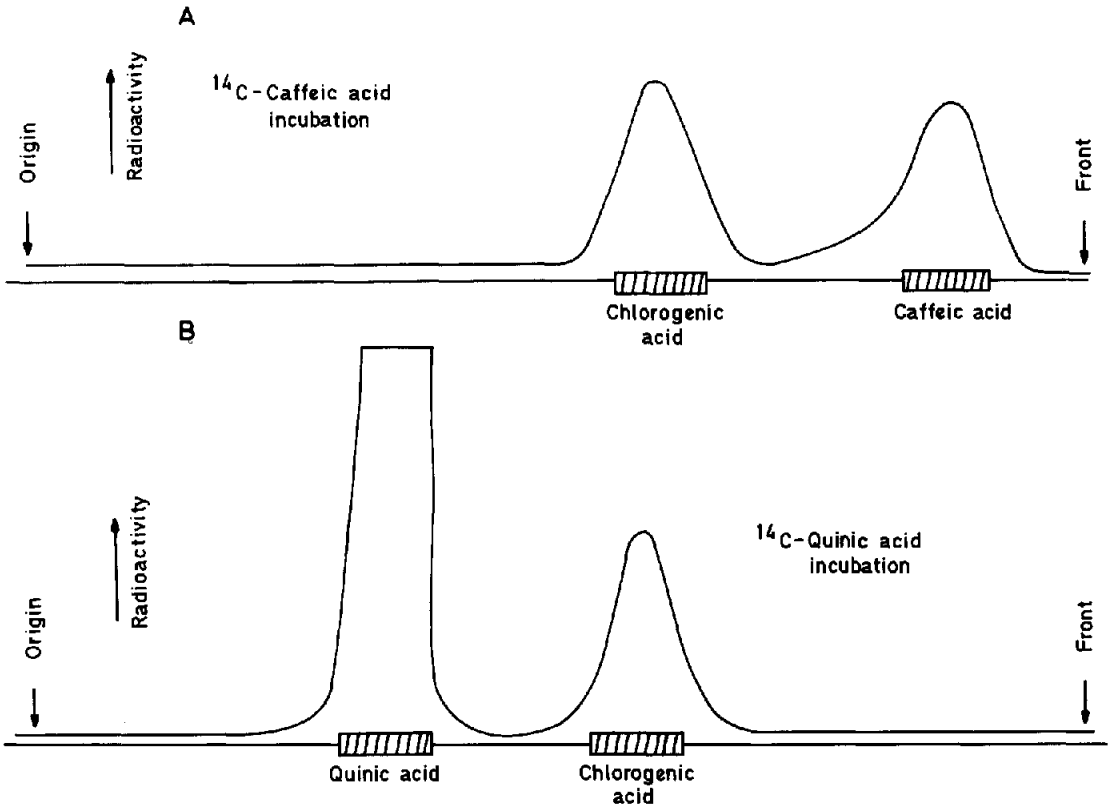

Fig. 1. Identification of chlorogenic acid by paper chromatography of a complete incubation mixture, as given in table 1. Labelled substrates were (A): [2-^{14}C]caffeic acid and (B): [G-^{14}C]quinic acid. Radioactivity was recorded with the thin layer scanner II (Berthold, Wildbad).

Table 1
Cofactor requirement for chlorogenic acid synthesis

System	Chlorogenic acid formed (μmoles) A	B
	from [2-^{14}C]-caffeic acid	from D-[G-^{14}C]-quinic acid
Complete	0.14	0.33
minus ATP	0.0	0.0
minus CoA	0.0	0.0
minus quinate	0.009	–
minus caffeate	–	0.0
minus enzyme	0.0	0.0

The reaction mixture contained in a final volume of 0.5 ml: 50 μmoles $KHPO_4^-$ pH 7.5, 5 μmoles $MgCl_2$, 2 μmoles dithiothreitol, 0.25 μmoles CoA-SH, 1.5 μmoles ATP, and in A) 0.3 μmoles caffeate (0.6 μCi), 5 μmoles quinate, 1.9 mg protein, in B) 2 μmoles quinate (0.6 μCi), 0.3 μmoles caffeate, 0.6 mg protein.

acid (25 mg) and acetylated using acetic anhydride pyridine [17]. Pentaacetylchlorogenic acid (mp/180–183°C from benzene, MS: M^+ 564 m/e) resulted with a specific activity of 894 dpm/μmol. This derivative was first recrystallized from: benzene (905 dpm/μmol); second from MeOH/H_2O (879 dpm/μmol); third from iso-PrOH/petrol. (861 dpm/μmol); fourth from : toluene/EtOH/*n*-hexane (870 dpm/μmol). Furthermore the labelled product was identical with an authentic sample of chlorogenic acid upon paper chromatography in six different solvent systems.

As is shown in table 1, the formation of chlorogenic acid is completely dependent on CoA and ATP. When labelled quinic acid was incubated with the protein fraction and the cofactors, the formation of chlorogenic acid was completely dependent on the presence of caffeate. However, incubation of [2-^{14}C]caffeate in the absence of quinate led to the formation of a small amount of chlorogenic acid. This formation of the ester was found to be due to the presence of trace amounts of quinic acid which were carried over from the growth medium into the protein fraction. After extensive dialysis of the protein fraction no chlorogenic acid was formed in the absence of quinate. The strict dependence of the formation of chlorogenic acid on the presence of both ATP and CoA suggested that caffeoyl-CoA is an intermediate in this reaction. Using the optical assay [15], hydroxy-cinnamate : CoA ligase was detected in this enzyme preparation. The activity with caffeate as substrate was found to be 1.2 nmole/min/mg protein under standard conditions [18].

In order to prove that caffeoyl-CoA is in fact the energy-rich intermediate in the formation of chlorogenic acid, synthetic caffeoyl-CoA was incubated in the presence and absence of quinate and the decrease in optical density at 345 nm (absorption of the caffeoyl-CoA thiol ester-linkage) observed. There is considerable cinnamoyl-CoA thiolesterase activity in this protein fraction (0.25 nmoles caffeoyl-CoA hydrolyzed per min per mg protein in 0.5 ml incubation mixture). However, in the presence of quinate, there is a more rapid decrease in optical density due to the transfer of the caffeoyl moiety of caffeoyl-CoA to quinate which corresponds to 0.60 nmol caffeoyl-CoA transfered per min. per mg protein in 0.5 ml. This result was substantiated by the finding that, in the presence of synthetic caffeoyl-CoA and [^{14}C] quinate, labelled chlorogenic acid was formed, as shown by paper chromatography. This reaction was seen only in the presence of protein. This proves that under these conditions caffeoyl-CoA functions as activated intermediate in the formation of chlorogenic acid (fig.2) and disproves that the formation of chlorogenic acid involves 'acetone insoluble' protein-bound cinnamic derivatives.

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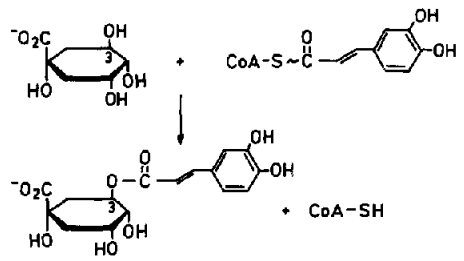

Fig. 2. Reaction sequence for the formation of chlorogenic acid.

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