

Hydroxycinnamoyl: Coenzyme A Transferase Involved in the Biosynthesis of Kaempferol-3-(*p*-coumaroyl Trigluconide) in *Pisum sativum*

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Acylated Flavonols, *p*-Coumaroyl: Coenzyme A, Hydroxycinnamoyl: Coenzyme A Transferase

The major flavonoids of *Pisum* are derivatives of kaempferol and quercetin, including both trigluconides and acylated trigluconides in which the acyl group is *p*-coumaric acid. Although hydroxycinnamic acid esters of flavonoids are common pigments in many plants, neither the enzymes nor the precursors involved in their biosynthesis have been demonstrated. We report here that crude enzyme preparations extracted from peas catalyze the transfer of the *p*-coumaroyl moiety of *p*-coumaroyl:Coenzyme A to kaempferol-3-trigluconide forming kaempferol-3-(*p*-coumaroyl trigluconide) as the acylated product. The reaction product has been vigorously shown to be identical to the naturally occurring kaempferol-3-(*p*-coumaroyl trigluconide) in both chromatographic and chemical properties. The enzymatic formation of the acylated derivative occurred only minimally when incubated with the cofactors required for carboxyl group activation (ligase) and maximally when incubated with *p*-coumaroyl: Coenzyme A as the acyl donor.

Introduction

The major flavonoids of *Pisum sativum* have been identified as kaempferol-3-trigluconide, quercetin-3-trigluconide and their acylated derivatives: kaempferol-3-(*p*-coumaroyl trigluconide) and quercetin-3-(*p*-coumaroyl trigluconide)^{1,2}.

The physiological role of these compounds has been extensively investigated and a correlation has been found between flavonol (kaempferol or quercetin) and the activities of phenylalanine ammonia-lyase, peroxidase and IAA oxidase³. It has also been shown that dark-grown pea seedlings produce only kaempferol derivatives whereas light-grown tissues produce both kaempferol and quercetin derivatives; moreover, different amounts of each are produced in individual tissues^{4,5}. The biosynthesis of flavonol derivatives in this plant has not been studied and little is known about the activities and specificities of the enzymes involved.

The presence of acylated flavonoids is widespread and acylation occurs mainly in the anthocyanin, flavonol and flavone class. Thus far, the acyl moiety has been found to arise from a number of organic acids⁶; however, the most predominant are those

belonging to the hydroxycinnamic acid class. To date, the acylated flavonoids have received limited attention and little is known about their biosynthesis. From recent studies on lignin^{7,8}, chlorogenic acid⁹, and flavonoid^{10,11} biosynthesis it has become well established that both condensation and carboxyl reduction of hydroxycinnamoyl compounds involves a high-energy thioester intermediate, and, therefore, it appears likely that these high-energy intermediates could also be involved in the formation of the acylated flavonols of peas. In support of this requirement for carboxyl group activation prior to acyl transfer, Hahlbrock¹² has reported the presence of a malonyl-CoA transferase, in cell suspension cultures of *Petroselinum*, which was involved in the biosynthesis of the acylated flavone glycoside, apin.

In this communication, we report the preparation of cell-free extracts from *Pisum sativum* seedlings, which catalyze the transfer of the *p*-coumaroyl moiety of *p*-coumaroyl-CoA to kaempferol-3-trigluconide to form kaempferol-3-(*p*-coumaroyl trigluconide).

Materials and Methods

Preparation of substrates. Kaempferol-3-trigluconide and kaempferol-3-(*p*-coumaroyl trigluconide) were isolated from hot water extracts of 15 day old pea seedlings grown under continuous light. The water fraction was concentrated and fractionated on a polyamide column (Macherey, Nagel and Co. SC-6 0.16 mm)¹³. The fractions were then chromato-

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Abbreviations: aKTG, Kaempferol-3-(*p*-coumaroyl trigluconide); KTG, Kaempferol-3-trigluconide; PCA, *p*-coumaric acid; PCA-CoA, *p*-coumaroyl:Coenzyme A.



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graphed on prewashed Whatman No. 1 in *tert*-butanol:acetic acid:water (3:1:1) (TBA) and 10% acetic acid. This combination of procedures yielded compounds which were chromatographically pure. To prepare stock solutions for enzyme assays, an extinction coefficient of $18 \times 10^6 \text{ cm}^2 \cdot \text{mol}^{-1}$ for kaempferol-3-triglucoside was used.

Radioactive *p*-coumaric acid was synthesized either by the condensation of [2- ^{14}C]malonic acid with *p*-hydroxybenzaldehyde or by deamination of [UL- ^{14}C]tryptophan with a phenylalanine ammonia-lyase enzyme preparation (Pabst Laboratories). [^{14}C]-*p*-coumaroyl-Coenzyme A was then synthesized according to a method provided by Prof. M. H. Zenk, Ruhr University, W. Germany (unpublished).

Enzyme extraction and assay. *Pisum sativum* L. var. Alaska were grown in continuous light for one week; then the seedlings were harvested, frozen in liquid nitrogen and powdered. An equal weight of wet polyclar AT was added and the mixture was extracted 45 min in 0.1 M borate buffer, pH 7.7 containing 10^{-2} M 2-mercaptoethanol. The homogenate was filtered through cheesecloth, centrifuged and the supernatant was fractionated with ammonium sulfate. The fraction between 30 and 70% was resuspended in 0.1 M phosphate buffer, pH 7.3, and was used as the enzyme source.

Reaction mixtures contained $0.2 \mu\text{mol}$ [^{14}C]-*p*-coumaroyl-CoA ($0.014 \mu\text{Ci}/\mu\text{mol}$), $0.189 \mu\text{mol}$ kaempferol-3-triglucoside, $50 \mu\text{mol}$ KH_2PO_4 pH 7.3, and approximately 1.5 mg of protein in a total volume of $400 \mu\text{l}$. Reactions were incubated 3 h at 30°C before being stopped by the addition of $800 \mu\text{l}$ absolute ethanol. The protein was removed by centrifugation, washed with 67% ethanol, re-centrifuged and the combined supernatants were chromatographed on prewashed Whatman No. 1 chromatograph paper in the TBA and 10% acetic acid solvents. Unlabelled kaempferol-3-(*p*-coumaroyl triglucoside) was added as carrier. Spots were located under UV at 366 nm and the kaempferol-3-(*p*-coumaroyl triglucoside) (R_F 0.51 in TBA and 0.52 in 10% acetic acid) was eluted with 80% ethanol. Radioactivity was determined by scintillation spectrometry (toluene containing 5.5 g PPO, 0.1 g POPOP and 333 ml of Triton X-100 in a total volume of one liter).

Protein was determined by the Lowry method¹⁴ as modified by Potty¹⁵.

Results and Discussion

Crude enzyme preparations from pea seedlings have been shown to catalyze the transfer of the *p*-coumaroyl residue of *p*-coumaroyl-CoA to kaemp-

ferol-3-triglucoside forming kaempferol-3-(*p*-coumaroyl triglucoside) as a reaction product. After incubation of [^{14}C]-*p*-coumaroyl-CoA with kaempferol-3-triglucoside and the enzyme preparation, a radioactive spot corresponding to this compound could be isolated. Fig. 1 shows the results of a two dimensional chromatogram from a complete reaction mixture. Of the 5 compounds found, three were radioactive. In this preparation approximately 20% of the total radioactivity was found associated with the acylated kaempferol-3-triglucoside. It is also

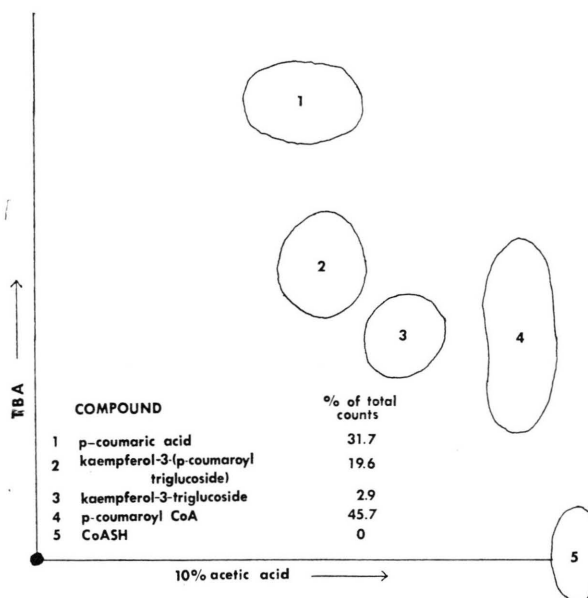


Fig. 1. Chromatographic separation of reaction constituents and distribution of radioactivity.

apparent that there was considerable thioesterase in the preparation as shown by the presence of a large amount of free *p*-coumaric acid liberated during incubation.

To prove the identity of the acylated kaempferol-3-triglucoside compound, a large reaction mixture was made and separated by chromatography on Whatman No. 1. The band corresponding to the acylated kaempferol-3-triglucoside was cut out, eluted, spotted on TLC plates or paper and chromatographed in four different solvents (TLC: chloroform:acetic acid:water (3:2:saturated) and *n*-butanol:acetic acid:water (6:1:2); paper; TBA and 10% acetic acid). In each case all the radioactivity applied was recovered in the acylated kaempferol-3-triglucoside spot.

The labelled product was further identified as follows: the eluted product was subjected to a mild base hydrolysis (1.0 N NaOH at room temp. 30 min), acidified to pH 1 with HCl and extracted with diethyl ether. The ether solubles, with unlabelled *p*-coumaric acid added as carrier, were chromatographed by TLC in the chloroform : acetic acid : water and 10% acetic acid solvents. The spots were located under UV, scraped off and the radioactivity determined. All of the radioactivity was located in the *p*-coumaric acid spot. Kaempferol-3-triglucoside was identified in the aqueous fraction.

As a final proof of the identity of the product, a chromatographically pure sample was acetylated¹⁶. The derivative was then chromatographed two dimensionally and the single spot was eluted with 95% ethanol. An aliquot (670 dpm) was subjected to a mild base hydrolysis, neutralized with HCl and chromatographed in CAW on microcrystalline cellulose. The radioactivity was recovered in two bands, one with chromatographic and UV properties identical to *p*-coumaric (416 dpm) and the remainder (190 dpm) in a bright blue band at the solvent front. This latter substance is probably a breakdown product from hydrolytic conditions and has been observed repeatedly during preliminary experiments with pure kaempferol-3-(*p*-coumaroyl triglucoside).

If the enzyme preparation was incubated in the presence of [¹⁴C]-*p*-coumaric acid, ATP, CoASH, Mg²⁺, kaempferol-3-triglucoside and a reduced

thiol, small amounts of the acylated flavonol were produced. (Table I A). This activity was completely dependent upon ATP and kaempferol-3-triglucoside. The low levels of activity detected when CoASH or Mg²⁺ were omitted were likely due to the endogenous levels of these compounds in the crude enzyme preparation. These results suggested that *p*-coumaroyl-CoA was an intermediate in this reaction. Using the optical assay¹⁷, hydroxycinnamate : CoA ligase activity was measured and found to be 1.1 nmol/min/mg protein under conditions described in Table I A.

The formation of acylated kaempferol-3-triglucoside was found to occur maximally when an enzyme preparation was incubated in the presence of synthetic *p*-coumaroyl-CoA and kaempferol-3-triglucoside. (Table I B). This product was not formed in the absence of *p*-coumaroyl-CoA, kaempferol-3-triglucoside or enzyme. The transferase activity was not altered by dialysis nor did pretreatment of the enzyme with an ion exchange resin¹⁸ result in any observable effect. Due to endogenous thioesterase activity, large amounts of free *p*-coumaric acid were also liberated, but this hydrolytic activity could be removed by chromatography on Sephadex G-200 and was also inhibited by the presence of kaempferol-3-triglucoside in the reaction mixture (Table I B). Thus, the increased efficiency of transfer observed in the presence of *p*-coumaroyl-CoA would indicate that this compound functions as an activated intermediate in the synthesis of acylated flavonols in peas. It is also one more example of the participation of this group of compounds in a variety of biological reactions.

Preliminary experiments have also indicated that the enzyme preparation catalyzes the transfer of the *p*-coumaroyl residue of *p*-coumaroyl-CoA to quercetin-3-triglucoside to form the acylated derivative. Presently, experiments are underway to further purify and characterize this activity. In view of the fact that kaempferol and quercetin compounds have been shown to be modulators of IAA oxidase extracted from peas^{2,19} and that these pigments are differentially synthesized in the tissues of light-grown or dark-grown plants²⁰, further characterization of the biosynthesis could provide a better understanding of their role in the growth and development of pea seedlings.

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Table 1. Cofactor requirements. [¹⁴C]PCA-CoA Complete System described in text. [¹⁴C] PCA Complete contained 0.114 μ mol [¹⁴C] PCA (50,000 dpm), 0.1 μ mol CoASH, 0.15 μ mol ATP, 0.15 μ mol Mg²⁺, 0.08 μ mol KTG and 10 μ mol KH₂PO₄ with approximately 0.12 mg of protein in a total volume of 100 μ l at pH 7.0. Incubation time: A — 1 h; B — 3 h: at 30 °C.

System	% of total counts		
	PCA-CoA	PCA	aKTG
A [¹⁴ C] PCA Complete	16	83	0.34
minus ATP	1.1	98.9	0.065
minus CoASH	0.45	99.4	0.17
minus Mg ²⁺	3.8	96	0.17
minus CoASH, ATP, Mg ²⁺	0.33	99.5	0.08
minus KTG	6.2	93.7	0.05
B [¹⁴ C] PCA-CoA Complete	38.5	46.3	13.5
minus KTG	26.5	73.6	0.001
minus enzyme	96.7	3.3	0

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