Enzymic Synthesis of Hydroxycinnamic Acid Esters of Glucaric Acid and Hydroaromatic Acids from the Respective 1-O-Hydroxycinnamoylglucoside and Hydroxycinnamoyl-Coenzyme A Thioester as Acyldonors with a Protein Preparation from *Cestrum elegans* Leaves

Dieter Strack*, Wiltrud Gross, Jürgen Heilemann, Harald Keller, and Sabine Ohm

Botanisches Institut der Universität zu Köln, Gyrhofstraße 15, D-5000 Köln 41, Bundesrepublik Deutschland

Z. Naturforsch. **43c**, 32–36 (1988); received August 25, 1987

Acyltransferase, Hydroxycinnamic Acid Conjugates, Glucaric Acid Ester, Quinic Acid Ester, Shikimic Acid Ester

Hydroxycinnamoyltransferase activities which catalyze the formation of O-hydroxycinnamoyl-(*p*-coumaroyl-, feruloyl-, and sinapoyl-)-glucaric acids *via* the corresponding 1-O-hydroxycinnamoyl-β-glucoses, and O-(*p*-coumaroyl)-quinic acid and O-(*p*-coumaroyl)-shikimic acid both *via* *p*-coumaroyl-CoA thioester have been isolated from leaves of *Cestrum elegans* D. F. L. v. Schlechtendal. The enzymic activities involved could be classified as 1-O-hydroxycinnamoyl-β-glucose:glucaric acid hydroxycinnamoyltransferase (EC 2.3.1.–) and *p*-coumaroyl-CoA:quinic acid/shikimic acid hydroxycinnamoyltransferase (EC 2.3.1.–). This is the first time that both the O-glucoside- and the S-CoA-dependent activities in phenolic acid-ester formation were found to be present in the same plant.

Introduction

It has recently been shown that in tomato cotyledons [1] and rye primary leaves [2] the biosynthesis of hydroxycinnamic acid (HCA)-glucaric acid O-esters is catalyzed by a HCA-quinic acid:glucaric acid HCA-transferase (in tomato) or by a HCA-coenzyme A (HCA-CoA):glucaric acid HCA-transferase (in rye). In the course of these studies our attention was drawn to *Cestrum* leaves, which exhibit metabolically active HCA-glucose (HCA-Glc) esters [3] indicating that they could possibly act as acyldonors in the formation of caffeoylglucaric acid in *C. euanthes* leaves [4]. Furthermore, results from time-course tracer studies with *C. poeppigii* leaves [3] were also discussed with respect to the possible HCA-Glc-dependent formation of HCA-quinic acid. This was shown to be realized in chlorogenic acid (5-O-caffeoylquinic acid) biosynthesis in the root of sweet potato [5, 6], an alternative to the widespread HCA-CoA-dependent formation [7, 8].

The biochemical-physiological role of the alternative pathways, involving the HCA-CoA thioester, the 1-O-(HCA)-acylglucoside as acyldonors [9], or – more indirectly – for example *via* quinic acid esters [1] leading to the vast array of phenolic acid conjugates in plants [10, 11], is presently under study in our laboratory. We report here on two alternative pathways, the enzymic formation of HCA-glucaric acids *via* the HCA-Glcs and of *p*-coumaroylquinic and *p*-coumaroylshikimic acids *via* the corresponding CoA thioester in *C. elegans* leaves. These results (i) extend the number of known 1-O-acyl-Glc-dependent acyltransferases [9, 12, 13] and (ii) show for the first time that the two major alternative pathways may be operative in the same plant.

Abbreviations: CoA, coenzyme A; Glc, glucose; HCA, hydroxycinnamic acid; HCA-, hydroxycinnamoyl (= hydroxycinnamic acid-ester moiety); HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

* *Present address:* Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, Mendelssohnstraße 1, D-3300 Braunschweig.

Reprint requests to Prof. Dr. D. Strack.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0100–0032 $ 01.30/0

Materials and Methods

Plant material

Young leaves of 3-year old *Cestrum elegans* D. F. L. v. Schlechtendal plants, grown in the greenhouse of the Cologne Institute, were used in the present study.

Substrates

HCA-Glcs came from petals of *Antirrhinum majus* (1-O-[*p*-coumaroyl]- and 1-O-feruloyl-β-Glcs) [14] and seedlings of *Raphanus sativus* (1-O-sinapoyl-β-Glc) [15], and were isolated as previously described [15]. Caffeoyl-Glc was not readily available. HCA-CoAs (*p*-coumaroyl-, caffeoyl-, feruloyl-, and sinapoyl-CoAs) were synthesized chemically by the ester-exchange reaction *via* the acyl-N-hydroxysuccinimide esters [16] and purified on polyamide (Perlon) columns [2]. D-Glucaric, D-galactaric, D-glucuronic, D-gluconic, and shikimic acids were purchased from Serva (Heidelberg, FRG). CoA (free acid), HCAs, D-quinic and chlorogenic acids came from Fluka (Neu-Ulm, FRG).

Protein preparation

Freshly harvested *C. elegans* leaves (10 g) were ground (15 min) in a mortar in the presence of 1 g quarz sand, 2 g insoluble Polyclar AT, and 50 ml Tris-HCl buffer (100 mM, pH 8.0) containing 20 mM DTT and 1 mM EDTA. The homogenate was passed through Miracloth and the filtrate centrifuged for 30 min at 20,000 × *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 30% saturation. The precipitate was removed by centrifugation and the supernatant was raised to 70% saturation. The precipitated protein was collected by centrifugation and redissolved in 3 ml potassium phosphate buffer (20 mM, pH 6.25) from which 2.5 ml was filtered through Sephadex G-25 (Pharmacia PD-10 columns). The eluate (3.5 ml) was used as the source of enzymic activity. Protein content (about 15 $mg \cdot ml^{-1}$) was determined by the method of Bradford [17] using bovine serum albumin as standard.

Enzyme assay and activity determination

The reaction mixtures contained in a total volume of 40 µl 15 mM potassium phosphate buffer (pH 6.25), 5 mM glucaric acid (or the other possible acceptors tested), 2–2.5 mM HCA-Glcs, and 149 µg protein. Control lacked glucaric acid. The reactions were started by the introduction of protein, stopped after incubation at 30 °C by the addition of 1 volume CH_3OH, and transferred to a freezer (−30 °C). The enzyme assay for possible chlorogenic acid-dependent reactions is described elsewhere [1]. The assays for the *p*-coumaroylquinic and -shikimic acid formation contained in a total volume of 40 µl 25 mM potassium phosphate buffer (pH 7.0), 5 mM DTT, 0.5 mM EDTA, 0.6 mM *p*-coumaroyl-CoA, and 25 mM quinic- or shikimic acid. Enzyme activities were determined by HPLC as described in Fig. 1 (Waters 600-pump system, 20 µl injection with an U6K injection system, and an UV/Vis-detector Waters 481; Waters-Millipore GmbH, Eschborn, FRG) and in Fig. 2 (LKB 2-pump system, 20 µl-loop injection *via* a Rheodyne system [Rheodyne Inc., Cotati, CA, USA], and a LKB UV/Vis detector model 2151; Pharmacia-LKB, Freiburg, FRG). Chromatograms and quantitative calculations were obtained with a Waters 740 data processor (Fig. 1) and a Shimadzu (Kyoto, Japan) Data Processor Chromatopac C-R3A (Fig. 2). Elution times in the system as described in Fig. 1: *p*-coumaroylglucaric acid, 15.5 min; feruloylglucaric acid, 16.9 min; sinapoylglucaric acid, 17.6 min; *p*-coumaroyl-Glc, 18.1 min; feruloyl-Glc, 19.1 min; sinapoyl-Glc, 19.7 min; *p*-coumaric acid, 21.9 min; ferulic acid, 23.4 min; sinapic acid, 24.0 min. Elution times in the system as described in Fig. 2: *p*-coumaroylquinic acid, 8.9 min; *p*-coumaroylshikimic acid, 12.9 min; *p*-coumaric acid, 11.2 min; *p*-coumaroyl-CoA, 14.3 min.

Identification of reactions products

The reaction products (HCA-glucaric acids) were isolated from the respective assays (scaled up to 1 ml) by polyamide (Perlon) column chromatography and identified by co-TLC of the alkaline (aq. KOH) hydrolysis products (HCAs and glucaric, quinic, and shikimic acids) as described previously [2].

Results and Discussion

When protein preparations from *C. elegans* leaves were incubated (i) with 1-O-(HCA)-β-Glcs (*p*-coumaroyl-, feruloyl-, and sinapoyl-Glcs) and glucaric acid and (ii) with *p*-coumaroyl-CoA and the hydroaromatic acids quinic and shikimic acids, a time-dependent appearance of O-HCA-glucaric acids and O-(*p*-coumaroyl)-quinic and O-(*p*-coumaroyl)-shikimic acids, respectively, were found on HPLC chromatograms. The course of transesterification was linear under the described conditions with the amount of added protein and with time up to at least 60 min for the Glc-dependent and 20 min

for the CoA-dependent acyltransferase. Heat-denatured protein (5 min at 90 °C) failed to catalyze these reactions. Fig. 1 and 2 illustrate the enzymic formations of O-sinapoylglucaric acid from 1-O-sinapoyl-β-Glc and glucaric acid and O-(*p*-coumaroyl)-quinic acid from *p*-coumaroyl-CoA and quinic acid, respectively. In all the assays run up to 60 min only one major product was formed indicating positional specificity of the ester formation. However, the position of the ester bond at the glucaric acid or the quinic acid moiety was not determined. Several isomers were described for the naturally occurring caffeic acid-glucaric acid ester from *C. euanthes* [4] and 5-O-(*p*-coumaroyl)-quinic acid as possible intermediate in the biosynthesis of chlorogenic acid (5-O-caffeoylquinic acid [18]) in *C. poeppigii* [19]. Co-HPLC with extracts from 6–7 day old cotyledons of *Lycopersicon esculentum,* which contain 2-O- or 5-O-caffeoylglucaric acid and chlorogenic acid as the sole major constituents at a ratio of about 1:1 [1], showed that 2-O- or 5-O-caffeoylglucaric acid in *C. elegans* leaves occurs as a minor compound and chlorogenic acid as a major one (HPLC analysis; not documented); also co-HPLC with the enzymatically

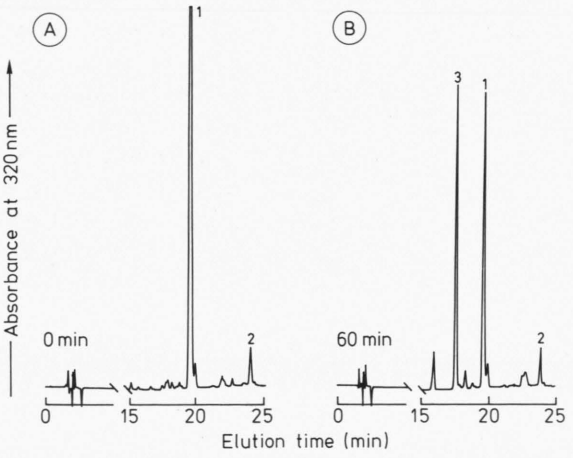

Fig. 1. Diagrams of HPLC analyses (0.128 absorbance units full scale; note break in axis) of a HCA-transferase assay with 1-O-sinapoyl-β-Glc and glucaric acid including 0.37% protein from *C. elegans* leaves immediately after introduction of protein (A, 0 min) and after 60 min (B) reaction time at 30 °C. Peak identification: 1 = 1-O-sinapoyl-β-Glc; 2 = sinapic acid; 3 = sinapoylglucaric acid. Chromatographic conditions: on prepacked Nucleosil C_{18} (5 µm, 250 × 4 mm *i.d.*; Macherey-Nagel, Düren, FRG) within 30 min from solvent A (1.5% H_3PO_4 in H_2O) to solvent B (1.5% H_3PO_4, 20% CH_3COOH, and 15% CH_3CN in H_2O) with a flow rate of 1.5 ml · min^{-1}.

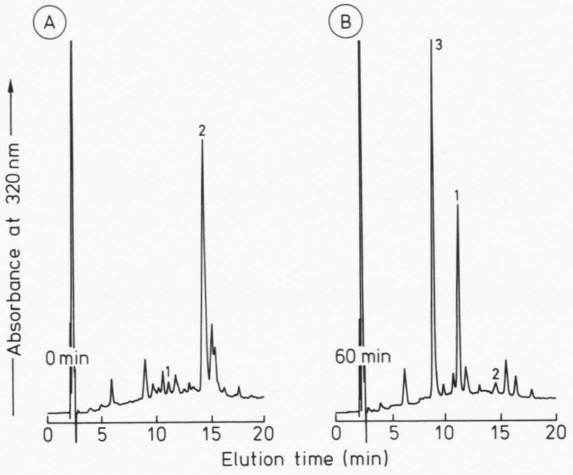

Fig. 2. Diagrams of HPLC analyses (0.064 absorbance units full scale) of a HCA-transferase assay with *p*-coumaroyl-CoA and quinic acid including 0.37% protein from *C. elegans* leaves immediately after introduction of protein (A, 0 min) and after 60 min (B) reaction time at 30 °C. Peak identification: 1 = *p*-coumaric acid; 2 = *p*-coumaroyl-CoA; 3 = *p*-coumaroylquinic acid. Chromatographic conditions: within 20 min from 30% solvent B to 70% B in (A + B); chromatographic column and solvents as described in Fig. 1.

formed products indicated the presence of small amounts of *p*-coumaroyl- and feruloylglucaric acids and *p*-coumaroylquinic acid (not further investigated).

The amounts of products formed within 1 h at 30 °C were found to be about 65, 50, and 33% glucaric acid esters of the respective feruloyl-, sinapoyl-, and *p*-coumaroyl-Glcs and within 20 min at 30 °C about 20% *p*-coumaroylquinic acid and about 50% *p*-coumaroylshikimic acid of the *p*-coumaroyl-CoA. These were identified by co-TLC of their alkaline hydrolysis products, *viz.* HCAs and glucaric, quinic, and shikimic acids, with reference material. The observed enzymic syntheses seem to be strictly acceptor specific. No products were formed with the HCA-Glcs when galactaric, glucuronic, gluconic, quinic, and shikimic acids were tested as possible acceptors or with chlorogenic acid (including only glucaric acid) as possible acyldonor [1]. This specificity is in agreement with previously studied analogous reactions (*e.g.* [20–23]). On the other hand, when we used the HCA-CoAs (including all used acceptors) we found a high transferase activity with *p*-coumaroyl-CoA and quinic and shikimic acids with a strict donor specificity. No products

could be detected (HPLC) either in short time assays (10 min) or in those run for 60 min) including caffeoyl-, feruloyl- or sinapoyl-CoAs as possible acyl-donors. However, the negative result with caffeoyl-CoA has to be treated with caution as we observed a rapid degradation (more than 90% within the first 10 min of incubation) of caffeoyl-CoA as well as the free caffeic acid in the enzyme assays (for possible mechanisms of such degradations see ref. [24–26]). We did not optimize a possible caffeoyl-CoA-dependent reaction. Whereas to date no HCA-Glc-dependent acyltransferase were found which showed a strict specificity for one acyldonor, the possible absolute specificity of the *p*-coumaroyl-CoA-dependent one is in agreement with some previous studies, *e.g.*, on the enzymic formation of *p*-coumaroylshikimic acid in *Cichorium endivia* [27]. Fig. 3 summarizes the results from the present study including the preceding "HCA-activating" UDP-Glc- and CoA-dependent reactions, which are widespread in the plant phenolic metabolism [9, 28]. The involved HCA-transferases could be classified as 1-O-(HCA)-β-Glc:glucaric acid HCA-transferase (EC 2.3.1.–) and *p*-coumaroyl-CoA:quinic acid/shikimic acid *p*-coumaroyltransferase (EC 2.3.1.–). The high activity of the shikimic acid-dependent transferase activity

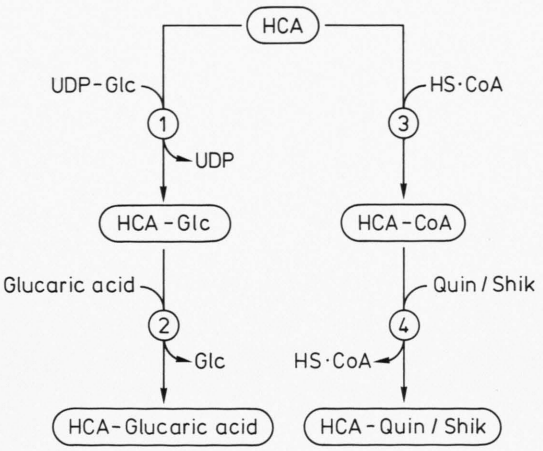

Fig. 3. Proposed general scheme for the enzymic synthesis of HCA-glucaric acids and hydroaromatic acids in *C. elegans* leaves. Enzymes involved: (1) = UDP-Glc:HCA Glc-transferase; (2) = HCA-Glc:glucaric acid HCA-transferase; (3) = HCA CoA-ligase; (4) = HCA-CoA:quinic acid/shikimic acid HCA-transferase (here *p*-coumaroyl-CoA:quinic acid/shikimic acid *p*-coumaroyltransferase; Quin = quinic acid, Shik = shikimic acid).

(about 2.5 times higher than the quinic acid-dependent one) and the fact that *p*-coumaroylshikimic acid could not be detected in the *C. elegans* leaves again raises the question [2, 27] on the metabolic role of shikimic acid conjugates in plants, possibly as acyl donor for an, as yet, unknown conjugation (esterification) reaction (*cf.* [27]) or further substitution of the phenylpropane moiety (hydroxylation and methylation) [29].

It is interesting to note, that we failed to show a HCA-Glc-dependent reaction in the formation of HCA-quinic acids, whereas the only quinic acid ester enzymically formed by the *C. elegans* protein preparation was found to be *p*-coumaroylquinic acid *via* *p*-coumaroyl-CoA. It has been suggested [3] that *p*-coumaroyl-Glc might be the pivitol intermediate in chlorogenic acid biosynthesis, excluding a major role for caffeic acid [19, 30], in *C. poeppigii* leaves. This could not be verified in the present study, although our results indicate the formation of chlorogenic acid *via* *p*-coumaroylquinic acid as suggested by Molderez *et al.* [3]. This hydroxylation reaction at the level of HCA O-esters has been repeatedly discussed, *e.g.*, in the formation of caffeoyltartronate in mung bean [22] and was shown with a microsomal preparation from parsley cell cultures in the enzymic formation of caffeoylshikimic acid [31].

In conclusion, this paper shows, in conjunction with our previous studies [1, 2], that the biosynthesis of HCA-conjugates, here HCA-glucaric acids, may proceed *via* different pathways, depending on the source of enzyme used. This is also true for biosynthesis of chlorogenic acid [5, 7]. Both are examples of converging lines of product formation. Fig. 4 summarizes the three pathways so far described leading to the accumulation of HCA-glucaric acids: the two widespread mechanisms *via* the HCA-CoAs, *e.g.* in rye primary leaves [2], and HCA-Glcs, *e.g.* in *C. elegans* leaves [this paper], as well as the more indirect pathway *via* quinic acid esters as shown with tomato cotyledons [1]. Also this is the first time that the two major alternative pathways for HCA-ester formation, the 1-O-acylglucoside- and the CoA thioester-dependent one, were shown to be operative in the same plant. It would be of great interest to investigate the role of this phenomenon, as a possible regulatory device for channelling the HCAs into different pathways. Compartmentation of these pathways at the tissue, cell or subcellular level should also be considered.

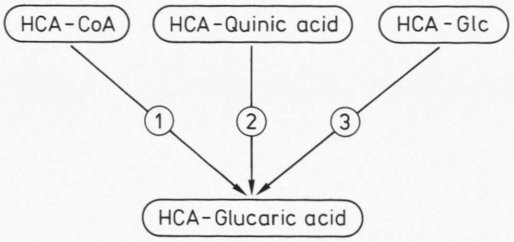

Fig. 4. Scheme for the three biosynthetic pathways of HCA-glucaric acids so far described in plants. Enzymes involved: (1) = HCA-CoA : glucaric acid HCA-transferase in primary leaves of *Secale cereale* [2]; (2) = HCA-quinic acid : glucaric acid HCA-transferase in cotyledons of *Lycopersicon esculentum* [1]; (3) = HCA-Glc : glucaric acid HCA-transferase in leaves of *C. elegans* [this paper].

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. Further support by a Heisenberg fellowship from the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

[1] D. Strack, W. Gross, V. Wray, and L. Grotjahn, Plant Physiol. **83,** 475–478 (1987).
[2] D. Strack, H. Keller, and G. Weissenböck, J. Plant Physiol. **131,** 61–73 (1987).
[3] M. Molderez, L. Nagels, and F. Parmentier, Phytochemistry **17,** 1747–1750 (1978).
[4] L. Nagels, W. Van Dongen, and F. Parmentier, Phytochemistry **21,** 743–746 (1982).
[5] R. J. A. Villegas and M. Kojima, Agric. Biol. Chem. **49,** 263–265 (1985).
[6] R. J. A. Villegas and M. Kojima, J. Biol. Chem. **261,** 8729–8733 (1986).
[7] J. Stöckigt and M. H. Zenk, FEBS Lett. **42,** 131–134 (1974).
[8] B. Ulbrich and M. H. Zenk, Phytochemistry **18,** 929–933 (1979).
[9] W. Barz, J. Köster, K.-M. Weltring, and D. Strack, in: Annual Proceedings of the Phytochemical Society of Europe (C. F. Van Sumere and P. J. Lea, eds.), **Vol. 25,** The Biochemistry of Plant Phenolics, pp. 307–347, Claredon Press, Oxford 1985.
[10] K. Herrmann, Fortschr. Chem. Organ. Naturstoffe **35,** 73–132 (1978).
[11] J. B. Harborne, in: Enzyclopedia of Plant Physiology, **Vol. 8,** Secondary Plant Products (E. A. Bell and B. C. Charlwood, eds.), pp. 329–402, Springer Verlag, Berlin 1980.
[12] D. Strack, J. Heilemann, B. Boehnert, L. Grotjahn, and V. Wray, Phytochemistry **26,** 107–111 (1987).
[13] D. Strack and M. Bokern, Planta, in press (1987).
[14] J. B. Harborne and J. J. Corner, Biochem. J. **81,** 242–250 (1961).
[15] D. Strack, Z. Pflanzenphysiol. **84,** 139–145 (1977).
[16] J. Stöckigt and M. H. Zenk, Z. Naturforsch. **30c,** 352–358 (1975).
[17] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[18] IUPAC, Biochem. J. **153,** 23–31 (1976).
[19] L. Nagels and F. Paramentier, Phytochemistry **15,** 703–706 (1976).
[20] D. Strack, Planta **155,** 31–36 (1982).
[21] D. Strack, W. Knogge, and B. Dahlbender, Z. Naturforsch. **38c,** 21–27 (1983).
[22] D. Strack, R. Ruhoff, and W. Gräwe, Phytochemistry **25,** 833–837 (1986).
[23] D. Strack, P. Leicht, M. Bokern, V. Wray, and L. Grotjahn, Phytochemistry **26,** 2919–2922 (1987).
[24] W. Barz and J. Köster, in: The Biochemistry of Plants, **Vol. 7,** Secondary Plant Products (E. E. Conn, ed.), pp. 35–84, Academic Press, London 1981.
[25] J. Negrel and T. A. Smith, Phytochemistry **23,** 31–34 (1984).
[26] J. Negrel and T. A. Smith, Phytochemistry **23,** 739–741 (1984).
[27] B. Ulbrich and M. H. Zenk, Phytochemistry **19,** 1625–1629 (1980).
[28] M. H. Zenk, in: Recent Advances in Phytochemistry, **Vol. 12,** Biochemistry of Plant Phenolics (T. Swain, J. B. Harborne, and C. F. Van Sumere, eds.), pp. 139–176, Plenum Press, New York 1979.
[29] T. Kühnl and E. Wellmann, Ber. Deutsch. Bot. Ges. **92,** 741–748 (1979).
[30] L. Nagels and F. Parmentier, Phytochemistry **13,** 2759–2762 (1974).
[31] W. Heller and T. Kühnl, Arch. Biochem. Biophys. **241,** 453–460 (1985).