Properties and Activity Changes of Chlorogenic Acid:Glucaric Acid Caffeoyltransferase From Tomato 
(Lycopersicon esculentum)\(^1\)

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ABSTRACT

A novel acyltransferase from cotyledons of tomato (Lycopersicon esculentum Mill.), which catalyzes the transfer of caffeic acid from chlorogenic acid (5-O-caffeoylquinic acid) to glucaric and galactaric acids, was purified with a 2400-fold enrichment and a 4% recovery. The enzyme showed specific activities (theoretical V\(_{\text{max}}\) per milligram of protein) of 625 nanokatals (caffeoyl-glucaric acid formation) and 310 nanokatals (caffeoyl-galactaric acid formation). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis it gave an apparent Mr of 40,000, identical to the value obtained by gel filtration column chromatography. Highest activity was found at pH 5.7, which was constant over a range of 20 to 120 millimolar K-phosphate. The isoelectric point of the enzyme was at pH 5.75. The reaction temperature optimum was at 38°C and the apparent energy of activation was calculated to be 57 kilojoules per mole. The apparent K\(_m\) values were 0.4 millimolar for glucaric acid, 1.7 millimolar for galactaric acid, and with both acceptors as second substrates 20 millimolar for chlorogenic acid. The relative ratio of the V\(_{\text{max}}\)/K\(_m\) values for glucaric acid and galactaric acid was found to be 100:12. Substratecompetition experiments support the conclusion that one single enzyme is responsible for both the glucaric and galactaric acid ester formation with marked preference for glucaric acid. It is proposed that the enzyme be called chlorogenic acid:glucaric acid O-caffeoyltransferase (EC 2.3.1.--). The three caffeic acid-dependent enzyme activities involved in the formation of the glucaric and galactaric acid esters, the chlorogenic acid:glucaric acid caffeoyltransferase as the key activity as well as the caffeic acid:CoA ligase and the caffeoyl-CoA:quinic acid caffeoyltransferase as the preceding activities, were determined. The time course of changes in these activities was followed during development of the seedling in the cotyledon and growth of the young plant in the first and second leaf. The results from tomato seedlings suggest a sequential appearance of these enzymes.

Although HCA\(^2\) conjugates, which occur in a vast array of primary and secondary compounds as ester moieties, are most widespread in the plant kingdom (6, 7), only a few of them have been investigated with regard to their enzymic synthesis. One reason this biochemical area has been neglected to date might be that since the discovery of the mechanism of the biosynthesis of a caffeic acid (3,4-bishydroxycinnamic acid) ester of quinic acid, chlorogenic acid (5-O-caffeoylquinic acid; 8), this has been widely accepted as the mechanism for all other known HCA esters. For Stöckigt and Zenk showed in 1974 (22) that in tobacco cell cultures the formation of chlorogenic acid proceeds via the caffeoyl-CoA thioester as acyl donor. However, it has been shown that beside this commonly found mechanism, 1-O-acyl glucosides, whose formation is catalyzed by UDP-glucose-dependent glucosyltransferases, may also act as HCA donors (1). Moreover, alternative esterifications (transacylations) may also be possible. For example, chlorogenic acid, which is common in Asteraceae, Solanaceae, and Rubiaceae (13) may act as an acyl donor molecule for caffeoyltransferases. This has been demonstrated by protein preparations catalyzing the formation of isochlorogenic acid, i.e. 3,5-bis-O-caffeoylquinic acid, in sweet potato (10, 37) and of caffeoylgalactaric and -galactaric acids in tomato (26).

In this paper, we report the purification and characterization of CGT from tomato. We present evidence that this enzyme is strictly donor specific in the acylation of glucaric and galactaric acids. Furthermore, we show that the time course of its activity, measured in cotyledons of the seedling and in the first and the second leaf of the young plant, correlates well with the accumulation patterns of caffeoylglucaric and -galactaric acids in these organs.

MATERIALS AND METHODS

Plant Material

Tomato (Lycopersicon esculentum Mill. cv Moneymaker Spezialzucht) seeds were obtained from Waltz (Stuttgart, FRG). Seedlings and young plants were grown as described earlier (26).

Chemicals and Substrates

With the exception of CM-Sepharose which came from Sigma (Deisenhofen, FRG) all the other column materials for enzyme purification were obtained from Pharmacia LKB (Freiburg, FRG). 3-O- and 4-O-cafeoylquinic acid were pro-

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\(^{2}\) Abbreviations: HCA, hydroxycinnamic acid; CGT, chlorogenic acid:glucaric acid caffeoyltransferase; CQT, caffeoyl-CoA:quinic acid caffeoyltransferase; FPLC, fast protein liquid chromatography.

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duced by heating (90°C) chlorogenic acid (Serva, Heidelberg, FRG), i.e. 5-O-cafeoylquinic acid (8), for 30 min in 200 mM K-phosphate at pH 7.0 (21). The isomers were separated by TLC on microcrystalline cellulose ('Avicel', Macherey-Nagel, Düren) twice in CHCl₃-CH₃COOH-H₂O (3:2, v/v, saturated with H₂O). The Rₛ after second development were 0.44, 0.50, and 0.59 for the 3-O-, 4-O-, and 5-O-isomer, respectively. The compounds were eluted from the cellulose powder with CH₃OH and purified by column chromatography on Sephadex LH-20 (90 × 2.5 cm i.d.) with 50% aqueous CH₃OH. The individual isomers were identified by TLC on silica gel (impregnated with KH₂PO₄) in ethylacetate-Me₂CO (4:1, v/v) with Rₛ of 0.35, 0.49, and 0.53 for the 3-O-, 5-O-, and 4-O-isomer, respectively (21), and by HPLC with retention times (min) of 6.0, 10.5, and 11.5 for the 3-O-, 4-O, and 5-O-isomer, respectively (c.f. ref. 12; old numbering system with the 3-O-isomer as chlorogenic acid). The 3-O-isomer of the 4-coumaric acid (4-hydroxycinnamic acid) ester of quinic acid, 3-O-(4-coumaroyl)-quinic acid, came from a phytochemical investigation on needles of Picea abies (L.) Karst. (31); the respective 5-O-isomer was produced via the CQT activity and the complete assay taken for testing possible CGT activity (substrate specificity). The sugar acids and free caffeic acid were purchased from Serva. Caffeoyl-CoA was synthesized chemically and identified according to Zenk and co-workers (5, 23) and purified on polyamide (perlon) columns (elution with 0.75 mM methanolic NH₃) by a method described elsewhere (28) with a yield of 40%.

**CGT Purification**

All purification steps were carried out at 4°C. Protein standard was determined by the Bradford method (3) using BSA.

**Step 1: Preparation of Crude Extract**

Frozen cotyledons (about 300 g stored at −20°C) from 8 to 10 d old tomato seedlings were homogenized in a Waring Blender in the presence of 1.5 L 100 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 10 mM DTT, and 30 g polyclar AT. The homogenate was passed through Miracloth and the filtrate centrifuged (30 min at 20,000g).

**Step 2: Ammonium Sulfate Precipitation**

The enzyme activity was precipitated by (NH₄)₂SO₄ (30–80% saturation) and redissolved in a minimal volume of 20 mM K-phosphate (pH 5.7). Excess (NH₄)₂SO₄ was removed by filtration through Sephadex G-25.

**Step 3: Ion Exchange on DEAE-Sephacel**

The protein solution from step 2 was chromatographed on a DEAE-Sephacel column (12 × 1.6 cm i.d.) at a flow rate of 54 mL·h⁻¹. The column was washed with 200 mL 20 mM K-phosphate (pH 6.0) before the following linear gradient was applied: 300 mL 0 to 300 mM KCl in 20 mM K-phosphate (pH 6.0). Active protein was concentrated by (NH₄)₂SO₄ precipitation (80% saturation) followed by centrifugation (30 min at 20,000g). The pellet was dissolved in a minimal volume of 20 mM Na-citrate (pH 5.0). This was filtered through Sephadex G-25.

**Step 4: Ion Exchange on CM-Sepharose**

The enzyme obtained from step 3 was applied to a CM-Sepharose column (25 × 1.6 cm i.d.). The column was washed with 120 mL 20 mM Na-citrate (pH 5.0) before the following linear gradient at a flow rate of 36 mL·h⁻¹ was applied: 400 mL 0 to 300 mM KCl in 20 mM Na-citrate (pH 5.0). Active protein was concentrated by (NH₄)₂SO₄ precipitation (80% saturation) and after centrifugation redissolved in a minimal volume of 50 mM K-phosphate (pH 7.0).

**Step 5: Molecular Exclusion on Ultrogel AcA 44**

The enzyme obtained from step 4 was applied to an Ultrogel AcA 44 column (95 × 1.6 cm i.d.). Protein was eluted with 50 mM K-phosphate (pH 7.0) at a flow rate of 15 mL·h⁻¹. Active protein was concentrated by ultrafiltration (PM 30 filter; Amicon, Witten, FRG).

**Step 6: Molecular Exclusion on FPLC-UltroPac TSK 3000 SW**

Sixty μL of the enzyme solution obtained from step 5 was applied to a FPLC-UltroPac TSK 3000 SW column (30 cm × 8 mm i.d.). Protein was eluted with 50 mM K-phosphate (pH 7.0) at a flow rate of 24 mL·h⁻¹. Active protein was concentrated by microconcentration (Centricon 10; Amicon) and subjected again to FPLC.

**Standard Enzyme Preparations**

Protein was prepared from seeds and dissected organs from seedlings and young plants essentially as described in steps 1 and 2 of the CGT purification procedure (see above) except that 40 seeds and young seedlings or 20 older seedlings and young plants were mixed with the extraction medium in a ratio of 1 g fresh weight:4 mL medium. The protein, precipitated between 30 to 80% (NH₄)₂SO₄ saturation, was redissolved in a minimum volume of 50 mM K-phosphate (pH 5.7) and desalted by filtration through PD-10 columns (Pharmacia LKB, Freiburg). The eluates (3.5 mL) were used as source of enzyme activities.

**Enzyme Assays and Activity Determinations**

**Caffeoyl-CoA Formation**

The reaction mixture contained in a total volume of 1 mL 100 mM Tris-HCl (pH 7.5), 0.5 mM caffeic acid, 0.2 mM CoA, 2.5 mM ATP, 2.5 mM MgCl₂, and up to 100 μL protein solution. The reaction was started by the addition of CoA. Enzymatic activity (at 30°C) was determined photometrically (18, 38) by following the increase in absorbance at 360 nm and applying the extinction coefficient of the CoA-ester determined by Gross and Zenk (5).

**Chlorogenic Acid Formation (19)**

The reaction mixture contained in a total volume of 50 μL 40 mM K-phosphate (pH 7.0), 0.05 mM caffeoyl-CoA, 2 mM...
quinic acid, 0.2 mM EDTA, 2 mM DTT, and 10 μL protein solution. The reaction (at 30°C) was started by the addition of protein and stopped after incubation of 10 to 30 min by transferring the mixture into liquid nitrogen. The enzymatic activity was determined by HPLC as follows: at a flow rate of 1 mL·min⁻¹ linear gradient elution from 20% solvent B within 7 min to 60% B in (A + B) followed by 2 min isocratically at 100% B (R of chlorogenic acid = 7.2 min).

Caffeoylglucaric Acid Formation

The reaction mixture contained in a total volume of 50 μL 20 mM K-phosphate (pH 5.7), 20 mM chlorogenic acid, 4 mM glucaric acid or galactaric acid, and 10 μL protein solution. The substrates were dissolved in 20 mM K-phosphate adjusted to pH 5.7. The reaction (at 30°C) was started by the addition of protein and stopped after incubation of 10 to 30 min by transferring the mixture into liquid nitrogen. The enzymatic activity was determined by HPLC as follows: at a flow rate of 1 mL·min⁻¹ linear gradient elution from 20% solvent B within 6 min to 26% B in (A + B) followed by 2 min isocratically at 100% B (Rs of caffeoylgalactaric acid and caffeoylglucaric acids = 4.6 and 5.4 min, respectively).

CGT Characterization

Mₐ Determination

The apparent Mr was determined on an Ultrogel AcA 44 column (see above) and by SDS-PAGE according to Neville and Glossman (16), and the proteins were detected by silver staining as described by Morrissey (14). The following reference proteins (Serva, Heidelberg) were used: myoglobin (Mr 17,800), chymotrypsinogen (Mr 25,000), ovalbumin (Mr 45,000), and BSA (Mr 67,000). Kₑ on Ultrogel column = (Vₑ - Vₐ)/(Vₑ - Vₐ) was estimated using Blue Dextran 2000 (Vₑ) (Pharmacia LKB, Freiburg) and Vₑ using DNP-alanine (Serva, Heidelberg). Rₛ from SDS-PAGE: myoglobin = 0.9, chymotrypsinogen = 0.65, ovalbumin = 0.44, and BSA = 0.29.

pH Optimum

The following buffer systems at 50 mM were used to determine the pH optimum of the CGT activity: Na-acetate (pH 4.0–5.0), Na-citrate (pH 5.0–6.6), K-phosphate (pH 5.3–7.1), and Tricine (pH 7.0–8.0). The actual pH values were measured after mixing the reaction assays.

Isoelectric Focusing

Determination of the isoelectric point was carried out on ‘Servalyt Precotes’ (pH 4–7; 125 × 125 mm; Serva, Heidelberg) at 3 W. After prefocus (prescription by Serva), 5 μL enzyme solution was loaded into the gel (62.5 mm from the left and right edge) and electrophoresis started at 500 V and terminated after 2 h at 1700 V.

Kinetic Properties

Apparent Kₑ and Vₑmax values at fixed concentrations of the second substrate were obtained by Lineweaver-Burk plots.

Substrate-competition experiments were carried out with varying glucaric acid concentrations (0.5, 1.0, 2.0, and 4.0 mM) in the presence of 1 mM galactaric acid and vice versa with varying galactaric acid concentrations in the presence of 1 mM glucaric acid.

Metabolite Extraction and Quantification

Metabolite extraction and quantification was done as described previously (26). Dissected organs from 2 seedlings or young plants at different stages of development were treated immediately after harvest with an Ultra-Turrax homogenizer in 5 to 20 mL 80% aqueous methanol (depending on organ size) for 4 to 5 min. The homogenates were allowed to stand for 30 min with continuous stirring and then centrifuged (10 min at 3000g). The clear supernatants were subjected to HPLC to separate and quantify the caffeic acid esters. Chromatographic conditions (standard program for rapid determination of the major metabolites) were: at a flow rate of 1 mL·min⁻¹ linear gradient elution from 20% solvent B within 15 min to 35% B (A + B) (Rs of caffeoylgalactaric acid, caffeoylgulcaric acid and chlorogenic acid: 4.6, 5.4, and 11.5 min, respectively); alternative extended program: as before but adding a second gradient-elution step from 35% solvent B in (A + B) within 10 min to 100% B.

HPLC

The liquid chromatograph (two-pump system, Pharmacia, LKB, Freiburg) was equipped with a prepacked 5 μm-Nucleosil C₁₈ column (25 cm × 4 mm i.d.; Macherey-Nagel, Duren) and the following solvents used: solvent A = 1.5% phosphoric acid in water; solvent B = 1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile in water. Injection was done via a Rheodyne rotary valve (Rheodyne Inc., Cotati, CA) with a 20 μL loop. Compounds were detected photometrically at 320 nm (LKB UV/VIS variable wavelength monitor) and quantified by external standardization (chlorogenic acid as reference compound) using a Shimadzu Data Processor Chromatopac C-R3A (Shimadzu Corporation, Kyoto).

RESULTS

CGT Isolation and Purification

The CGT from 8 to 10 d old tomato cotyledons was purified 2400-fold with a yield of 4% according to the procedure listed in Table 1. The final theoretical Vₑmax activity obtained was 624 nkat·(mg protein)⁻¹ in the formation of caffeoylglucaric acid and 310 nkat·(mg protein)⁻¹ in the formation of caffeoylgalactaric acid. Both crude protein preparations and the purified enzyme could be stored for several months at –20°C with no apparent loss of activity. However, refreezing and thawing resulted in a loss of activity of 20 to 30%.

General CGT Properties

The formation of caffeoylglucaric and -galactaric acids was positively correlated with protein and was linear with time up to 60 min with 164 μg protein·(mL assay)⁻¹ from the standard crude preparation and up to 10 min with 0.2 μg purified
Table I. Purification of CGT from Tomato Cotyledons

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity*</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nkat</td>
<td>mg</td>
<td>nkat. mg⁻¹</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>122</td>
<td>1245</td>
<td>0.098 100</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄</td>
<td>75</td>
<td>658</td>
<td>0.114 61.5</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>3. DEAE-Sephacel</td>
<td>47</td>
<td>35.7</td>
<td>1.73 38.5</td>
<td>13.5</td>
<td>1</td>
</tr>
<tr>
<td>4. CM-Sephacel</td>
<td>22.7</td>
<td>1.7</td>
<td>17.5 88.6</td>
<td>179</td>
<td>1</td>
</tr>
<tr>
<td>5. AcA 44-Ultrogel</td>
<td>10.8</td>
<td>0.21</td>
<td>51.4 8.8</td>
<td>524</td>
<td>1</td>
</tr>
<tr>
<td>6. FPLC-Ultropac (twice)</td>
<td>5.2</td>
<td>0.022</td>
<td>236.4 4.3</td>
<td>2412</td>
<td>1</td>
</tr>
</tbody>
</table>

* Standard assay conditions.

enzyme (mL assay⁻¹). Product identification was performed previously by chromatographic comparison with the in vivo compounds (26).

Both the CGT activity from crude protein preparations and the purified enzyme had the same elution volume from AcA 44 columns, giving a Mr of about 40,000. An identical value was obtained by slab gel SDS-PAGE. Some protein preparations, especially from older seedlings (from 14 d onward, not documented), showed a second major protein band on electrophoresis with an Mr of about 513,000. The facts that in preparations from younger seedlings (e.g. 8 d old) the 153 kD protein could not be detected, that substrate specificities of the CGT activity did not differ in these preparations from younger and older seedlings, and that glucaric and galactaric acids act as competitive inhibitors (see below) indicated that a single protein (Mr of 40,000) is responsible for the described enzymatic activity.

The effect of pH on the catalytic property of CGT was tested from pH 4.0 to 8.0. The pH optimum was found to be at pH 5.7 in K-phosphate with 50% of this activity near pH 4.2 in Na-acetate and pH 6.2 in K-phosphate. The maximal activity was constant between 20 and 120 mm K-phosphate with near 50% of this activity at 300 mm. Isoelectric focusing of the purified enzyme showed a single protein band with an isoelectric point of pH 5.75.

The effect of temperature on enzyme activity was tested from 10 to 60°C. The maximal rate of product formation was found to be near 38°C. From these data the apparent energy of activation was calculated from a linear Arrhenius plot to be 57 kJ mol⁻¹.

The addition of SH-group reagents (e.g. DTT or 2-mercaptoethanol) to the assay mixture did not increase enzyme activity nor did the SH-group inhibitor p-chloromercuribenzoate affect the rate of product formation.

CGT activity was slightly enhanced by the addition of 1 mm each of Mg²⁺ (MgSO₄, MgCl₂) and Ca²⁺ (CaCl₂) to 125 and 150% activity, respectively, compared with the control without divalent cations. However, concentrations above 5 mm resulted in a marked activity inhibition reaching about 50% at 40 mm. The addition of EDTA showed no effect.

Substrate Specificity and Kinetics of CGT

As in the first study (26), a position-specific reaction was found, since there was only one product formed from chlorogenic and glucaric acids, showing chromatographic identity with the naturally occurring 2-O- or 5-O-cafeoylglucaric acid and the galactaric acid ester. As possible donors, three caffeoylquinic acid isomers (3-O, 4-O, and 5-O) and two 4-coumaroylquinic acid isomers (3-O and 5-O) were tested, and as possible acceptors, glucaric, galactaric, gluconic, glucuronic, galactonic, and galacturonic acids were tested. The results are shown in Table II. The enzyme was strictly specific for 5-O-cafeoylquinic acid (chlorogenic acid) as acyl donor and glucaric and galactaric acids as acyl acceptor molecules. Thus, in summary, there is both a hydroxycinnamic acid (caffeic acid)-specific and an isomer (chlorogenic acid)-specific caffeic acid transfer to the two aldaric acids tested. Experiments with isolated caffeoylglucaric acid (26), designed to show possible free reversibility of the transferase reaction, were unsuccessful.

The reactions showed, in the ranges tested, typical Michaelis Menten-saturation curves with increasing substrate concentrations (5-6 different concentrations of chlorogenic, glucaric and galactaric acids). Double-reciprocal plots according to Lineweaver and Burk were linear and gave apparent Kₘ values of 0.4 mm for glucaric acid [R² = 0.992] and 1.7 mm for galactaric acid [R² = 0.947] both at a fixed concentration of the second substrate (20 mm chlorogenic acid). A respective value of about 20 mm for chlorogaric acid was found with fixed concentrations (8 mm) of both acceptors as the second substrates (glucaric acid: R² = 0.937; galactaric acid: R² = 0.918). The Kₘ values were not dependent on the concentration of the second substrate (not documented). The relative ratio of V_max/Kₘ values for glucaric acid and galactaric acid was found to be 100:12.

The fact that both reactions are most likely catalyzed by a single protein is not only indicated by its general properties described above, but also by the observation that both glucaric acid and galactaric acid acted as competitive inhibitors when added as the second substrate to the enzyme assays (Fig. 1). As expected, this substrate-competition experiment showed highest affinity of the enzyme toward glucaric acid (more effective displacement of galactaric acid by glucaric acid than vice versa). This corresponds well with the V_max/Kₘ values of the acceptors.

Table II. Substrate Specificity of CGT Activity

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Acceptor</th>
<th>V_max/Kₘ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Glucaric acid</td>
<td>1560</td>
</tr>
<tr>
<td>4-O-Caffeoylquinic acid</td>
<td>Glucaric acid</td>
<td>—</td>
</tr>
<tr>
<td>3-O-Caffeoylquinic acid</td>
<td>Glucaric acid</td>
<td>—</td>
</tr>
<tr>
<td>5-O(4-Coumaroyl)-quinic acid</td>
<td>Glucaric acid</td>
<td>—</td>
</tr>
<tr>
<td>3-O(4-Coumaroyl)-quinic acid</td>
<td>Glucaric acid</td>
<td>—</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Galactaric acid</td>
<td>182</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Gluconic acid</td>
<td>—</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Glucuronic acid</td>
<td>—</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Galactonic acid</td>
<td>—</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>Galacturonic acid</td>
<td>—</td>
</tr>
</tbody>
</table>

* V_max with glucaric acid = 624 nkat. (mg protein)⁻¹; V_max with galactaric acid = 310 nkat. (mg protein)⁻¹; Kₘ of the acylated acceptors: glucaric acid = 0.4 mm and galactaric acid = 1.7 mm.  
<sup>a</sup> No reaction according to HPLC analysis.
CGT FROM TOMATO

Time Course of Changes in Enzyme Activities Involved in the Formation of Caffeoylglucaric and -Galactaric Acids

Figures 2 and 3 illustrate the time course of changes in the pivotal enzyme activities involved in the formation of caffeoylglucaric and -galactaric acids starting from free caffeic acid, i.e. caffeic acid:CoA ligase, CQT, and CGT. In addition, the patterns of product accumulation in tomato cotyledons and leaves are shown. (The HPLC analyses with the extended program [see “Materials and Methods”] revealed besides the chlorogenic, caffeoylglucaric and -galactaric acids several additional unidentified UV-absorbing [phenolic] compounds in very low concentrations.) All three organs showed a rapid increase in CGT activity which correlated well with the accumulation patterns of the major HCA ester, caffeoylglucaric acid. The CGT activity could routinely be determined (standard assays) only at suboptimal chlorogenic acid concentrations (20 mM). Thus, the activity values shown are appreciably lower than the theoretical V_{max} values.

The cotyledons revealed sequential rapid activity increases of the three enzymes, which fit well with the proposed pathway (Fig. 2). The activities per pair of cotyledons of the CoA ligase and CQT transiently increased showing maximal activities of 19 pkat at d 4 and 15 pkat at d 5, respectively, with highest
Figure 3. Accumulation patterns of metabolites (chlorogenic acid, caffeoylglicaric acid, and caffeoylgalactaric acid) and time course of changes in the involved enzyme activities (caffeoyl-CoA ligase, CQT, and CGT) from the first and second tomato leaf. Data (mean of two determinations) are calculated on the basis of one leaf. For further details see Figure 2.

Table III. Specific Activities of Caffeic Acid:CoA Ligase, CQT, and CGT from Tomato Cotyledons and Leaves

<table>
<thead>
<tr>
<th>Organ</th>
<th>Ligase</th>
<th>CQT</th>
<th>CGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons (d 17)</td>
<td>31</td>
<td>34</td>
<td>188</td>
</tr>
<tr>
<td>Leaf 1 (d 56)</td>
<td>21</td>
<td>20</td>
<td>283</td>
</tr>
<tr>
<td>Leaf 2 (d 56)</td>
<td>14</td>
<td>17</td>
<td>235</td>
</tr>
</tbody>
</table>

* Standard assay conditions (mean of three determinations).

rates of activity increase at stages of a rapid accumulation of the chlorogenic acid level. Subsequently, the CGT activity appeared and increased up to 16 pkat concomitantly with increases in the levels of caffeoylglicaric acid as the major component and caffeoylgalactaric acid as the minor one. This phenomenon suggests it would be interesting to study the regulation of this pathway (sequential substrate inductions?).

In contrast to the cotyledons, the leaves show a more or less simultaneous appearance of the three enzymes, low activities of the CoA ligase and CQT, reaching 6 to 7 pkat per leaf, and high activities of CGT, reaching 90 and 240 pkat from the first and second leaf, respectively (Fig. 3). The specific activities of the enzymes (pkat-[mg protein]⁻¹) gave essentially the same patterns of activity changes (not documented) and reached comparable values (same order of magnitude) in the three organs investigated (Table III).

**DISCUSSION**

It is shown in the present paper that tomato plants contain an acyltransferase which uses specifically the family—(Solanaceae)—characteristic chlorogenic acid (13) as the acyl donor in the formation of caffeoylglicaric acid and -galactaric acids. A corresponding reaction has been described in the formation of isochlorogenic acid, *i.e.* 3,5-bis-O-caffeoylquinic acid in sweet potato (10, 37). So far, this is the third mechanism found in plants responsible for the formation of HCA O-esters besides the following more commonly described mechanisms:

(a) *via* the HCA-CoA thioesters, *e.g.* in the formation of O-esters with quinic acid (22), shikimic acid (35), lupinine (15, Strack D, Becher A, Brall S, Witte L, unpublished data), tartaric acid (25), isocitric acid (29), various sugar acids (28), 3,4-bishydroxylactic acid (17), and 13-hydroxylupanine (Strack D, Becher A, Brall S, Witte L, unpublished data) as well as with flavonoids, flavonol glycosides (20), and anthocyanins (9);

(b) *via* the 1-O-acyl-HCA glucosides, *e.g.* in the formation of O-esters with malic acid (33), choline (24), 1-O-sinapoyl-glucose in a 'disproportionation' reaction (4), quinic acid (11), tartaric acid (27), and glucaric acid (30) as well as with betacyanins, namely betanin and amaranthin (2).

These alternative pathways are not exclusively dependent on the nature of the conjugating moiety, but on the source of enzyme used, *i.e.* the plant investigated. The biosynthesis of HCA glucaric acid is a good example of converging lines for the formation of HCA O-esters. In rye primary leaves, it proceeds *via* the HCA-CoA thioester (28), in *Cestrum elegans* leaves *via* the 1-O-acyl-HCA glucoside (30), and, for the present work, in tomato leaves *via* the O-acyl-HCA quinic acid, here chlorogenic acid. The biosynthesis of chlorogenic acid itself is another example of such alternative pathways. It may be synthesized *via* the caffeoyl-CoA, *e.g.* in tobacco cell cultures (22) and various other systems (34) including the tomato plant or *via* the 1-O-caffeoylglucose, until now only found in sweet potato roots (36).

It will be of great interest (a) to search for further possible alternative acyltransfer reactions in plants, which may result in the discovery of various other mechanisms for such biosynthetic routes, and (b) to evaluate the reason and the importance of such a biochemical convergence in the metabolism of HCA conjugates. Concerning the latter, phylogenetic differentiations of biosynthetic routes as well as biochemical control mechanisms at different (sub)cellular locations should be kept in mind.

**LITERATURE CITED**

4. Dahlbender B, Strack D (1984) Enzymatic synthesis of 1,2-disinapoylglucose from 1-sinapoylglucose by a protein prepa-


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