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HYDROXYCINNAMIC ACID ESTERS OF ISOCITRIC ACID: ACCUMULATION AND ENZYMATIC SYNTHESIS IN *AMARANTHUS CRUENTUS*

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Key Word Index—*Amaranthus cruentus*; Amaranthaceae; hydroxycinnamic acid; caffeic acid; tricarboxylic acid; isocitric acid; ester.

Abstract—New hydroxycinnamic acid esters have been isolated from the cotyledons of *Amaranthus cruentus*. (*E*)-Caffeoylisocitric acid was identified as the major constituent and *p*-coumaroyl- and feruloylisocitric acids as minor ones on the basis of ^{1}H and ^{13}C NMR spectroscopy. FAB mass spectrometry, high-performance liquid and thin-layer chromatography. The structure of caffeoylisocitric acid was confirmed by chromatographic comparison with synthetic material. The accumulation of the new hydroxycinnamoylisocitric acids and their enzymatic synthesis via the hydroxycinnamoyl-CoA thioesters as acyl donors are described. The caffeoyl-CoA-dependent acyltransferase showed a rapid transient increase in activity reaching *ca* 5 pkat per cotyledon pair (i.e. *ca* 390 pkat per mg protein) at day six of seedling development.

INTRODUCTION

Among a vast array of naturally occurring hydroxycinnamic acid esters [1, 2] there are many examples of conjugates containing aliphatic (hydroxy)carboxylic acids (depsides), e.g. the frequently found tartaric acid derivatives [3]. There is only one example of a tricarboxylic acid as an ester moiety in hydroxycinnamic acid conjugates, hydroxycitric acid bound to *p*-coumaric, caffeic and ferulic acids in *Zea mays* [4].

In this communication we furnish evidence for new hydroxycinnamic acid tricarboxylic acid esters, viz. *p*-coumaroyl-, caffeoyl- and feruloylisocitric acids in seedlings and adult plants of *Amaranthus cruentus*. Furthermore their accumulation kinetics and enzymatic syntheses in cotyledons were studied and found to proceed via the hydroxycinnamic acid-coenzyme A thioesters as acyl donors. Development of the caffeoyl-CoA-dependent enzyme activity correlated well with the *in vivo* accumulation pattern of caffeoylisocitric acid.

RESULTS AND DISCUSSION

Structures of betalains and hydroxycinnamic acid esters and their accumulation

HPLC analyses of 50% aqueous methanolic extracts of cotyledons from *Amaranthus* seedlings (Fig. 1) showed two major secondary phenolic compounds, the betacyanin amaranthin, betanidin 5-*O*-glucuronosylglucoside

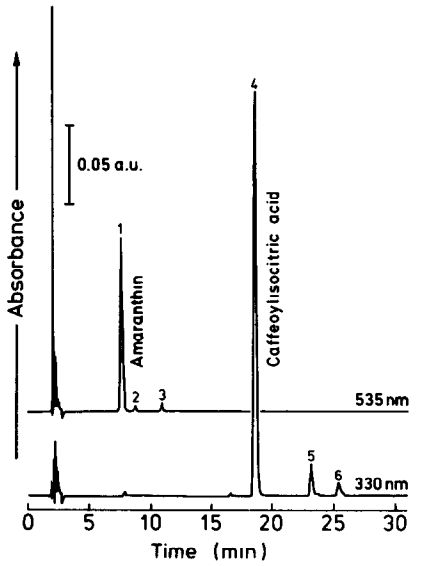

Fig. 1. HPLC analyses of secondary products (peak 4, caffeoylisocitric acid, R_t 18.7 min and peak 1, amaranthin, R_t 7.8 min) from cotyledons of 3-week-old *Amaranthus cruentus* seedlings. The very minor peaks 2 and 3 at 535 nm are isomaranthin (R_t 8.8 min) and betanin (R_t 11.1 min), while the minor peaks 5 and 6 at 330 nm are *p*-coumaroylisocitric acid (R_t 23.1 min) and feruloylisocitric acid (R_t 25.6 min). The chromatographic column used was prepacked with Nucleosil C_{18} (5 μm) (Macherey-Nagel, Düren, F.R.G.), 250 × 4 mm. Chromatographic conditions: linear gradient elution within 30 min from 10 to 70% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) at a flow rate of 1 ml/min.

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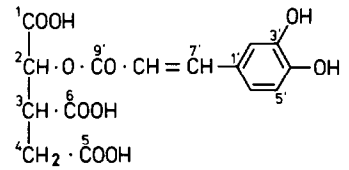

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(*ca* 95% of total absorbance at 535 nm), and the hydroxycinnamic acid ester caffeoylisocitric acid (1) (*ca* 85% of total absorbance at 330 nm, i.e. *ca* 27 nmol per cotyledon pair). The molar ratio of caffeoylisocitric acid: betanin, reached at the stationary phase of the pattern of caffeoylisocitric acid accumulation (Fig. 2), was *ca* 5:1. The adult plant contained in their young and older leaves 5–10% and 10–20% caffeoylisocitric acid, respectively, among a complex pattern of UV absorbing (330 nm) unknown phenolic compounds (hydroxycinnamic acid conjugates and flavonoids).

The known amaranthin was readily identified from the crude extract by direct chromatographic comparison with reference betacyanins, extracted from *A. tricolor* [5]. In addition, isoamaranthin and betanin were identified as minor constituents at *ca* 2 and 3% of total absorbance at 535 nm, respectively. For isolation and structure elucidation of 1, the crude methanol extract was prefractionated on a polyamide (Perlon) column. 1 was eluted with 0,1% ammoniacal methanol, isolated by TLC on microcrystalline cellulose in CAW, obtained in a pure form by column chromatography on Sephadex LH-20 in methanol and was subjected to NMR and MS spectroscopy. The chromatographic behaviour (CC, TLC) and detection of 1 on TLC under UV (350 nm) with and without treatment of ammonia vapour gave the first indication, as to its nature as a caffeic acid ester. In addition, two minor compounds, *p*-coumaroyl- and feruloylisocitric acids, were detected and characterized by UV spectroscopy. 1, caffeoylisocitric acid, and the minor esters, *p*-coumaroyl- and feruloylisocitric acids showing *ca* 7 and 3% of total absorbance at 330 nm (Fig. 1), respectively, were identified by chromatographic comparison of the hydrolytic products with authentic hydroxycinnamic acids (TLC in TAW) and isocitric acid (TLC in PFW and BAW).

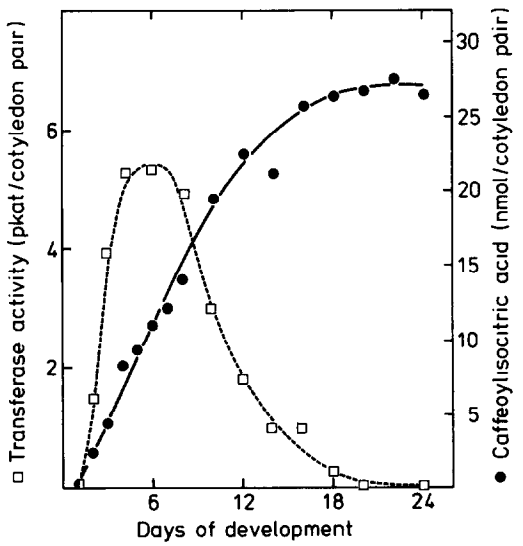

Fig. 2. Development of transferase activity (□, caffeoyl-CoA as acyldonor and isocitric acid as acceptor) and the accumulation pattern of caffeoylisocitric acid (●) in growing cotyledons of *Amaranthus cruentus;* mean data from two separate preparations with duplicate determinations.

^{1}H NMR data at high field allowed identification of the spin systems in 1, while the ^{1}H and ^{13}C chemical shifts provided information as to the positions of the various substituents in the molecule. Mass spectral data gave the M_r and the fragmentation ions confirmed the presence of both moieties in the molecule. These data, in conjunction with the chromatographic studies allowed the structure of 1 to be unambiguously determined. This was substantiated by comparison with synthetic caffeoylisocitric acid and the enzymatically formed isocitric acid conjugates (see below).

Enzymatic synthesis of isocitric acid conjugates

Protein preparations from cotyledons of *A. cruentus* were assayed for the enzymatic synthesis of hydroxycinnamoylisocitric acids. Two known mechanisms of synthesis via CoA- and glucose-carboxyl-group activation [6] were tested and it was found that the ester formation proceeds exclusively via the hydroxycinnamoyl-CoAs as the activated donors. The amounts of hydroxycinnamoylisocitric acids formed were positively correlated with the amount of protein and were linear for 10–15 min at 30°. The reactions were freely reversible, which was proved by photometrical analyses and HPLC (cf. ref. [7]). We propose the following reaction mechanism:

hydroxycinnamoyl-CoA + isocitric acid ↔ hydroxycinnamoylisocitric acid + HS·coenzyme A

The enzymatically formed products were identified by co-chromatography (TLC, HPLC) with reference samples of CoA and the isocitric acid hydroxycinnamates. Heat denatured (5 min at 80°) protein showed no enzymatic activity. The protein preparation showed no loss of enzymatic activity when stored at −20° for several weeks.

The reaction with hydroxycinnamoyl-CoAs and isocitric acid showed hyperbolic saturation curves with increasing substrate concentrations. Eadie–Hofstee plots [8, 9] gave apparent K_m values of 45, 55 and 163 μM for the respective caffeoyl-, *p*-coumaroyl- and feruloyl-CoAs (at 15 mM isocitric acid) and 208, 257 and 412 μM for isocitric acid in the presence of caffeoyl-, *p*-coumaroyl- and feruloyl-CoAs (0.2 mM each). There was only trace activity with sinapoyl-CoA as donor (*ca* 0.2% of the reaction velocity obtained with caffeoyl-CoA). The relative ratios of the V_{max}/K_m values for caffeoyl-, *p*-coumaroyl- and feruloyl-CoAs were 100:90:47), respectively. These enzymatic activities exhibited a strict specificity towards isocitric acid. Citric, malic, tartaric (*m*, L, R) and quinic acids tested, were not accepted as possible substrates.

The pattern of transferase development (Fig. 2) showed a rapid transient increase in activity reaching *ca* 5 pkat per cotyledon pair (i.e. *ca* 390 pkat per mg protein) at day six of seedling development. This correlates well with the accumulation pattern of caffeoylisocitric acid. The enzyme was not detected until day two of germination and extractable activity disappeared in cotyledons of *ca* 3-week-old seedlings. Young leaves from the adult and flowering plants also showed appreciable activities of the enzyme(s) (data not shown). In conclusion, the CoA-dependent biosynthesis of hydroxycinnamoylisocitric

acids in *A. cruentus* is another example of the important role of hydroxycinnamoyl-CoAs in the phenylpropanoid metabolism of higher plants [10].

EXPERIMENTAL

Plant material. Seeds of *A. cruentus* L. cv. Öschberg (*A. paniculatus* L., *A. chlorostachys* Willd., *A. hybridus* L., *A. hybridus ssp. paniculatus* (L.) Hejny) were purchased from Walz Samen GmbH, Stuttgart, F.R.G. and plants were grown in a greenhouse for preparative work and in a phytotron for analytical extraction and kinetic studies. The latter was carried out in a defined soil (type T, Balster, Fröndenberg, F.R.G.) under the following conditions: fluorescent light (*ca* 10000 lx) with a 14 hr day at 24–25° and 70% rel. humidity. Flowering plants were grown in the botanic garden of the Cologne institute.

Preparative extraction and isolation. Cotyledons of *Amaranthus* seedlings were homogenized in a Waring Blendor in 50% aq. MeOH (150 g fr. wt/200 ml) and then allowed to stand for 1 hr with continuous stirring. The suspension was filtered and the clear extract evapd to dryness *in vacuo* and the residue suspended in a few ml of H_2O. The known constituent amaranthin was readily identified by comparison with ref. material with the aid of thin-layer electrophoresis [11] and HPLC [12]. 1 was isolated by polyamide CC (CC-6 Perlon, 4.5 × 33 cm) (Macherey–Nagel, Düren, F.R.G.). After transfering the extract onto the H_2O-equilibrated column, 1.5 l H_2O, 1.5 l MeOH, 1 l Me_2CO and 1.5 l MeOH were applied as solvents. Subsequent elution with 2.5 l 0.1% NH_4OH in MeOH gave 1 as the major and *p*-coumaroyl- and feruloylisocitric acids as minor compounds. The latter fraction was evapd to dryness and the residue suspended in 5ml 50% aq. MeOH which was kept for further analyses. CC elution was monitored continuously by UV absorption at 254 nm. 1 was chromatographed on microcrystalline cellulose layers (20 × 20 cm) ('Avicel', Macherey–Nagel) in CAW ($CHCl_3$–HOAc, 3:2, H_2O satd), detected under UV at 350 nm (bright blue fluorescence changing to greenish-blue when treated with NH_3 vapour), scraped off and eluted with 50% aq. MeOH. After re-chromatography on a polyamide column (2 × 24 cm), 1 was finally purified on a Sephadex LH-20 column (3 × 53 cm) (Pharmacia, Uppsala, Sweden) using MeOH as solvent. It exhibited the following R_f values: on microcrystalline cellulose in CAW 0.14; in PFW (*n*-pentanol–HCO_2H–H_2O; 6.1:6.1:0.3) 0.47; in BAW (*n*-BuOH–HOAc–H_2O; 6:1:2) 0.71. *p*-Coumaroyl- and feruloylisocitric acids, changing from dark absorption to dark blue and from blue to greenish-blue fluorescence, respectively, when treated with NH_3 vapour, gave R_fs of 0.3 and 0.45 in CAW, 0.62 and 0.66 in PFW, 0.83 and 0.78 in BAW. The UV spectra in 50% aq. MeOH gave the following λ_{max} and bathochromic shifts after the introduction of 1 drop of 0.1 M aq. NaOH to 1 ml solution: 1, 328 nm (+42 nm); *p*-coumaroylisocitric acid, 310 nm (+44 nm); feruloylisocitric acid, 326 nm (+47 nm).

Analysis of hydrolysis products. 1, *p*-Coumaroyl- and feruloylisocitric acids were treated with 0.5 M aq. NaOH (pre-treated with a stream of N_2) for 30 min at room temp. TLC of the neutralized hydrolysate (Dowex 50 WX8, Serva, H^+) showed products identical with caffeic, *p*-coumaric and ferulic acids in TAW (toluene–HOAc; 2:1, H_2O satd), R_fs 0.18, 0.54, 0.67, respectively, and with isocitric acid in PFW, R_f 0.26 (citric acid, 0.29), and BAW, R_f 0.47 (citric acid, 0.50).

Analytical extraction and quantification. Cotyledons from 15–30 seedlings at different development stages were homogenized with an Ultra-Turrax homogenizer in 2 ml 50% aq. MeOH for 2–3 min. The homogenate was allowed to stand for 1 hr in the dark and was then centrifuged at 3000*g* for 10 min. The clear supernatant was subjected to HPLC to separate and quantify compound 1 and amaranthin.

HPLC. The liquid chromatograph and data processor are described elsewhere [7]. Injection was performed via a Rheodyne rotary valve (Rheodyne Inc., Cotati, CA, U.S.A.) with a 20 μl loop. For details of the chromatographic conditions see Fig. 1. Chlorogenic acid (Fluka, Neu-Ulm, F.R.G.) was used as an ext std for quantification of compound 1, for that of amaranthin an ε value ($cm^{-1} \times mol^{-1}$) was used of λ_{max}^{MeOH} nm (log ε): 535 (4.75) [13].

NMR and MS. ^1H and ^{13}C NMR spectra were recorded at ambient temp. at 400 and 100 MHz, respectively, locked to the major deuterium resonance of the solvent, CD_3OD. Chemical shifts were recorded for a mixture of the *E*- and *Z*-isomer of 1 (*ca* 9:1).

Negative ion FAB-MS were recording using glycerol as matrix.

(E)-*Isomer of caffeoylisocitric acid* (1). ^1H NMR (CD_3OD): δ7.636 [*d*; H-7′; J (7′-8′) 15.9 Hz], 7.105 (*d*; H-2′; J (2′-6′) 2.0 Hz], 6.993 [*dd*; H-6′; J (6′-5′) 8.2 Hz], 6.821 [*d*; H-5′], 6.373 [*d*; H-8′], 5.377 [*d*; H-2; J (2-3) 5.0 Hz], 3.462 [*ddd*; H-3; J(3-4A) 9.6, J(3-4B) 4.6 Hz], 2.835 [*dd*; H-4A; J(4A-4B) (−)16.8 Hz], 2.661 [*dd*; H-4B]. ^{13}C NMR (CD_3OD): δ = 176.03, 175.78, 174.29 (*s* x3; C-1, C-5, C-6), 168.39 (*s*, C-9′), 149.56, 146.77 (*s* × 2; C-3′, C-4′), 147.40 (*d*, C-7′), 127.97 (*s*, C-1′), 123.03 (*d*, C-6′), 116.28 (*d*, C-5′), 115.34, 115.05 (*d* × 2; C-2′, C-8′), 75.31 (*d*, C-2), 45.68 (*d*, C-3), 34.67 (*t*, C-4). Negative ion FAB MS *m/z* (%): 445 (9) $[M-H+glycerol]^-$, 353 (41) $[M-H]^-$, 191 (100) $[HO_2C{\cdot}CH(O){\cdot}CH(CO_2H){\cdot}CH_2CO_2H]^-$, 173 (66) $[191-18]^-$.

(Z)-*Isomer of caffeoylisocitric acid* (1). ^1H NMR (CD_3OD): δ = 7.498 [*d*; H-2′; J (2′-6′) 2.0 Hz], 7.082 [*dd*; H-6′; J (6′-5′) 8.3 Hz], 6.842 [*d*; H-7′; J (7′-8′) 12.9 Hz], 6.765 [*d*; H-5′], 5.871 [*d*; H-8′], 5.341 [*d*; H-2; J (2-3) 5.1 Hz], 3.416 [*ddd*; H-3; J (3-4A) 9.7, J (3-4B) 4.5 Hz], 2.799 [*dd*; H-4A], 2.623 [*dd*; H-4B]. ^{13}C NMR (CD_3OD): signals of C-1, C-2, C-4, C-5 and C-6 are identical with those of the (*E*)-isomer. δ = 167.41 (*s*, C-9′), 145.55 (*d*, C-7′), 128.19 (*s*, C-1′), 125.24 (*d*, C-6′), 118.52 (*d*, C-5′), 116.28, 115.81 (*d* × 2, C-2′, C-8′), 45.55 (*d*, C-3). Signals for C-3′ and C-4′ were too weak to be observed.

Synthesis of (E)-*caffeoylisocitric acid and CoA thioesters.* Caffeoylisocitric acid was chemically synthesized according to our previously published procedure [14] via the the caffeic acid chloride, produced by means of oxalyl chloride, and the hydroxycinnamoyl-CoAs via the acyl *N*-hydroxysuccinimide esters [15].

Enzyme preparation and assay. Protein was prepd from cotyledons of *A. cruentus* seedlings by a described procedure [7]. Kinetics of transferase forward reaction: The reaction mixture contained in a total vol of 50 μl 50 mM K-Pi (pH 7), 10 mM DTT, 1 mM EDTA, various amounts of *p*-coumaroyl-, caffeoyl-, feruloyl- and sinapoyl-CoAs (0.01–0.27 mM), 0.1–1 mM isocitric acid, and 5 μl protein soln (≃ 35 μg protein). Reactions were started by the introduction of hydroxycinnamoyl-CoA or isocitric acid and were stopped after incubation at 30° for 10 min by transfering the mixtures to a freezer (−20°) or by immediate HPLC analyses. *Transferase reverse reactions:* The mixt. contained in a total vol. of 0.75 ml 65 mM K-Pi (pH 7), 13 mM DTT, 1.3 mM EDTA, 0.2 mM hydroxycinnamoyl isocitric acid, 0.4 mM CoA and 70 μl protein soln. The reaction was started by the introduction of CoA.

Determination of enzyme activities. Transferase reverse reactions were optically assayed (increase in A through formation of the CoA-thioesters [15–17]. The forward reactions were determined by means of HPLC using linear gradient elution within 10 min from 40 to 60% solvent B in A; see Fig. 1 for further details. Apparent K_m and V_{max} values at fixed concns of

the second substrate were obtained by Eadie–Hofstee plots [8, 9] with 5 or 6 points.

Development of enzyme activity was determined by the reverse reaction. Cotyledons from 50 seedlings were processed in duplicate at different stages of growth. Protein content was determined by the method of ref. [18] using BSA as standard.

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