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# HYDROXYCINNAMOYL-COENZYME-A:TARTRONATE HYDROXYCINNAMOYLTRANSFERASE IN PROTEIN PREPARATIONS FROM MUNG BEAN

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**Key Word Index**—*Phaseolus radiatus*; Fabaceae; mung bean; hydroxycinnamic acid; tartronic acid; depside; biosynthesis; phenylpropanoid metabolism; hydroxycinnamoyl-CoA:tartronate hydroxycinnamoyltransferase.

**Abstract**—A CoA-thioester dependent transferase, hydroxycinnamoyl-CoA:tartronate hydroxycinnamoyltransferase, involved in the formation of hydroxycinnamoyltartronic acids, was extracted from young plants of mung bean and some of its properties determined. Occurrence and development of the transferase in different organs of the young plant correlated well with the accumulation patterns of the hydroxycinnamoyltartronates.

## INTRODUCTION

As recently described, the mung bean accumulates as major secondary products *p*-coumaroyl- and caffeoyl-tartronates in all of the overground organs of the young plant [1]. To date two major mechanisms for the formation of such esters are known, the first proceeds via CoA thioesters and the second via 1-*O*-acyl glucosides, being synthesized through activities of acyl-CoA ligases and UDP-glucose dependent transferases, respectively [2]. We studied these two mechanisms of the enzymatic synthesis of the tartronic acid conjugates in mung bean and report here on their formation via the CoA thioesters.

## RESULTS AND DISCUSSION

Protein preparations from different organs of young plants of mung bean were assayed for the enzymatic synthesis of *p*-coumaroyl-, caffeoyl- and feruloyl-tartronate. This was done with hydroxycinnamoyl-CoAs and tartronic acid and alternatively—including activity of CoA ligase—with free CoA, free hydroxycinnamic acids, ATP and tartronic acid. It was found that the formation of the tartronate conjugates proceeds exclusively via the corresponding thioesters (Fig. 1). No product was formed when 1-(*p*-coumaroyl)-glucose was tested as possible acyl donor.

In order to characterize some of the enzyme properties, the transferase activity was partially purified on a column of DEAE-cellulose resulting in a 28-fold purification. In agreement with previous work on CoA-thioester dependent hydroxycinnamoylester synthesis [3–5] the esterification reactions leading to the hydroxycinnamoyl-tartronates were freely reversible. The forward reaction was determined using *p*-coumaroyl- and caffeoyl-CoA and the back reaction using *p*-coumaroyl-, caffeoyl- and feruloyltartronates as donors. Both reactions were positively correlated with protein and were linear with time up to *ca* 10 min at 30°. Maximal enzymatic activities were found to be at pH 7.0 in KPi buffer and were dependent on the presence of a thiol compound (DTE) and EDTA. There was no appreciable difference in initial reaction velocities when measured the forward and back reactions. For example a protein preparation from primary leaves of 4-day-old plants showed specific activities (per mg protein) of 69 pkat *p*-coumaroyltartrate and 67 pkat *p*-coumaroyl-CoA in the forward and reverse reactions, respectively. Since the CoA thioesters were not readily available in sufficient quantities we routinely assayed this enzymatic activity using the tartronate conjugates and CoA as the substrates. Figure 2 illustrates the determination of this reaction by means of HPLC.

Table 1 shows the substrate specificity of the reaction using the three tartronic acid conjugates from mung bean, tartrate and malate derivatives isolated from spinach and radish, respectively, and 3-caffeoylquinate (chlorogenic acid) from a commercial source. As seen the back reaction of the enzymatic activity exhibited a strict specificity towards tartronate and a pronounced specificity towards the *p*-coumaric acid moiety. The ratios of the apparent $V_{max}/K_m$ values for *p*-coumaroyl-, caffeoyl- and feruloyltartronate were found to be 100:15:2, respectively, and it is interesting to note that the ratios of the reaction velocities showed no appreciable differences when determined in protein extracts from the different organs of mung bean (Table 2): *p*-coumaroyl-/caffeoyltartronate and *p*-coumaroyl-/feruloyltartronate were in the range of 3–4 and 6–8, respectively. There was no difference when protein from organs at different developmental stages were used (data not shown). These results, however, have to be interpreted with caution. It might be that the substrate affinities ($K_m$ values) in the forward reaction could appreciably differ from those of the back reaction [see 4]. The $K_m$ value towards CoA was found to be independent of the hydroxycinnamoyltartronate structure as second substrate. In the presence of *p*-coumaroyl- and caffeoyltartronate the $K_m$ values (mean from four independent determinations each) were 61 and 63 $\mu$M, respectively.

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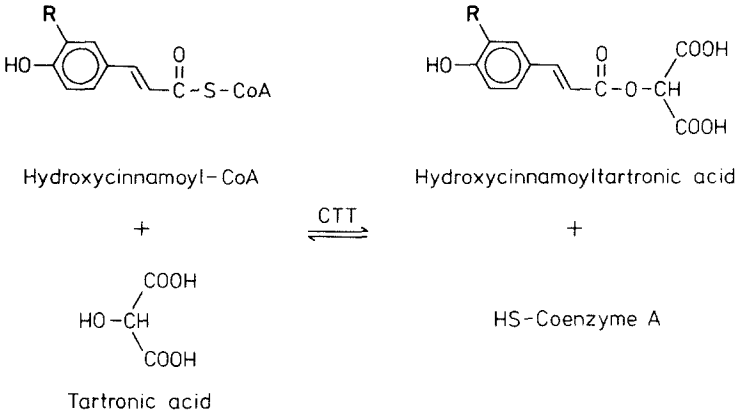


Fig. 1. Reaction scheme for the formation of hydroxycinnamoyltartronates catalysed by hydroxycinnamoyl-CoA : tartronate hydroxycinnamoyltransferase (CTT) in mung bean. **R** = H = *p*-coumaroyl; **R** = OH = caffeoyl; **R** = OMe = feruloyl conjugate.

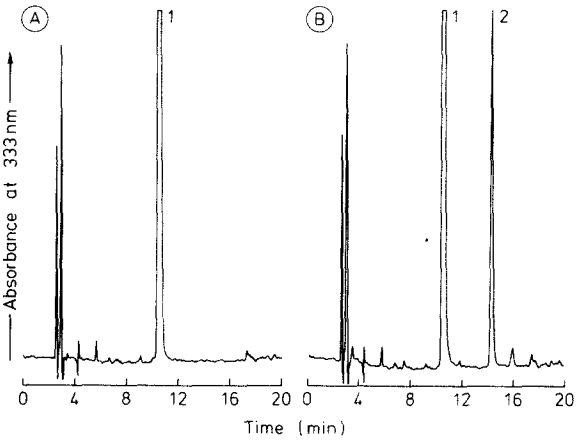


Fig. 2. HPLC analyses of transferase assays (reverse reaction) without (A) and with CoA (B). 1 = *p*-coumaroyltartronate; 2 = *p*-coumaroyl-CoA; when present, free sinapic acid elutes at 11.6 min. Chromatographic conditions: linear gradient elution within 20 min from 30 to 70% solvent B (1.5% $H_3PO_4$, 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. Detection was at 0.032 absorbance unit full scale. The column was prepacked with Shandon ODS Hypersil 5 μm, 250 mm long and 4.6 mm inner diameter.

Table 1. Substrate specificity (reverse reaction) of the transferase preparation from 6-day-old mung bean primary leaves

| Substrate | % Relative activity | $K_m$ (μM) |
|---|---|---|
| *p*-Coumaroyltartronate | 100* | 11 |
| Caffeoyltartronate | 26 | 20 |
| Feruloyltartronate | 13 | 65 |
| *p*-Coumaroyltartrate | 0 | — |
| Acetyl-(*p*-coumaroyl)-tartrate | 0 | — |
| *p*-Coumaroylmalate | 0 | — |
| 3-Caffeoylquinate | 0 | — |

* $V_{max}$ (102 pkat CoA thioester/mg protein).

Figure 3 illustrates the development of the transferase activities (standard enzyme preparation) in all the four overground organs of mung bean. As seen there are rapid transient increases in activity which correlated well with the accumulation patterns of *p*-coumaroyl- and caffeoyl-tartronic acids. The third tartronate conjugate present, the feruloyltartronate [1], was always found in minute quantities (data not shown). We proved that also the root accumulated *p*-coumaroyltartronate (82 nmol per individual root system at day 6) and it exhibited a transferase activity of about 5.0 pkat/root (76 pkat/mg protein).

Considering that the primary leaves accumulate caffeoyltartronate whereas the other organs show *p*-coumaroyltartronate as the major compound [1, this paper Fig. 3] the present results of the enzymatic activities (see above) could indicate that there might be one enzyme population whose *in vivo* activities were determined by different amounts of the individual substrates available, i.e. the primary leaf possibly supplies mainly caffeoyl-CoA whereas the other organs predominantly supply *p*-coumaroyl-CoA. A similar situation was recently discussed to occur in the 1-acylglucose dependent synthesis of *p*-coumaroyl- and caffeoylmalic acids, the major compounds in radish leaves. Here the involved enzyme shows highest specificity towards sinapoylglucose as acyl donor [6].

A second possible explanation for the discrepancy between transferase specificity and the accumulation pattern of tartronic acid conjugates in the primary leaves might be the involvement of a hydroxylase, acting on *p*-coumaroyltartronate to form the caffeoyl conjugate. Using a phenolase preparation [7, 8] from mung bean primary leaves, we found that there was an appreciable hydroxylase activity towards *p*-coumaroyltartronate, amounting to 233 pkat caffeoyltartronate/mg protein in 6-day-old primary leaves. This is about 60% of the phenolase activity which was found with free *p*-coumaric acid as substrate. It has to be born in mind that phenolase reactions in plants exhibit broad specificities and their role is not yet clearly understood [9, 10] and it has been difficult to establish the role of this enzyme *in vivo* [11]. However, there is supporting evidence for an important role of *p*-coumaric acid hydroxylase activity [11] and the caffeoyl-CoA derivative being the common acyl donor in the synthesis of caffeoyl depsides [4, 12].

Table 2. Substrate specificities (velocities of reverse reactions; pkat CoA thioester/mg protein) of transferase preparations from the different organs of the mung bean

| Organ | Coum-Tar* | Caf-Tar | Fer-Tar | Coum-Tar per Caf-Tar | Coum-Tar per Fer-Tar |
|---|---|---|---|---|---|
| Primary leaf (6)† | 102 | 27 | 13 | 3.8 | 7.8 |
| Cotyledon (3) | 96 | 28 | 15 | 3.4 | 6.4 |
| Hypocotyl (6) | 46 | 17 | 7 | 2.7 | 6.6 |
| Epicotyl (9) | 34 | 9 | 6 | 3.8 | 5.7 |

*Coum-Tar, *p*-coumaroyltartronate; Caf-Tar, caffeoyltartronate; Fer-Tar, feruloyltartronate.

†Day of development.

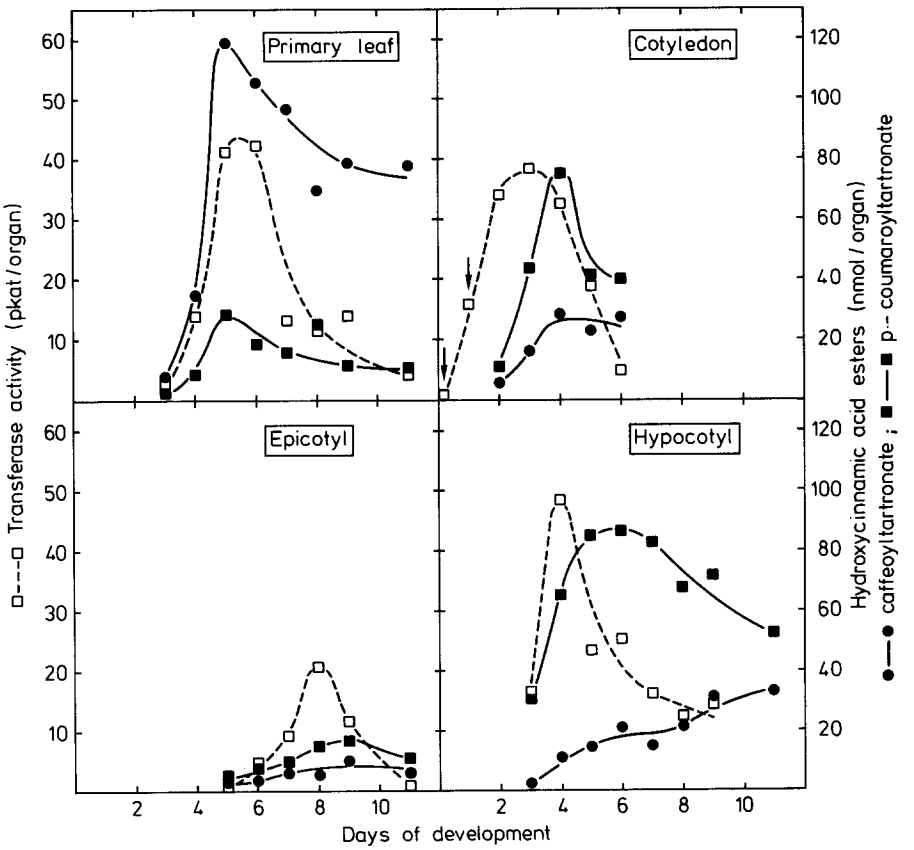


Fig. 3. Accumulation patterns of *p*-coumaroyl- and caffeoyltartronates and kinetics of transferase activities (back reaction with *p*-coumaroyltartronate and CoA as substrates) in different organs of mung bean. Arrows in the upper right graph (cotyledon) indicate transferase activities derived from the dry seed (day 0) and intact germinated seed (day 1), respectively.

It has been repeatedly discussed that in case of caffeoyl conjugates a second pathway leading to the dihydroxylation pattern may exist. There is evidence that *ortho*-hydroxylation of *p*-coumaric acid can proceed at the level of CoA thioester [13] or the depside derivative [14]. In our previous paper on hydroxycinnamoyltartronates [1] we discussed that this second possibility may be realized in mung bean and it has been suggested on the basis of inhibition studies with the fungal toxin tentoxin [15] that *p*-coumaric acid hydroxylase is not involved in the formation of caffeic acid in this plant. We made the 'tentoxin-experiment' according to Duke and Vaughn [15] and reproduced their results. Tentoxin-treated plants showed total chlorosis, shown by Duke and associates to be caused by photobleaching and inhibition of protochlorophyll(ide) accumulation [16, 17]. Our phenolase preparation from these plants showed only traces of hydroxylase activities towards free *p*-coumaric acid as well

as towards *p*-coumaroyltartronate, i.e. almost 100% inhibition compared to the control plants, grown under identical culture conditions. As in the study of Duke and Vaughn [15], we found that the accumulation of the caffeoyltartronate in the primary leaves was not affected at all. (Also the accumulation of rutin and nicotiflorin [1] was not affected.) It is interesting to note that in 5-day-old dark-grown plants there was in the primary leaves a marked decrease in the accumulation rate of caffeoyltartronate (*ca* 50%), whereas the level of *p*-coumaroyltartronate was increased from 30 to *ca* 60 nmol per leaf. However, the extractable activity of the phenolase preparation showed no difference compared to the light-grown control plants. This result supports the view of Duke and Vaughn that phenolase is not involved in the *ortho*-hydroxylation of phenolic compounds in mung bean. It will be of great interest to solve this problem of *ortho*-hydroxylation. Experiments have been initiated in this laboratory to isolate and characterize the hydroxylase involved.

## EXPERIMENTAL

*Plant material.* Young plants of *Phaseolus radiatus* L. (= *Vigna radiata* (L.) R. Wilczek = *Phaseolus aureus* Roxb.) ('Katjang Idjoe', Conimex Baarn, The Netherlands) were grown in a defined soil (type T, Balster, Fröndenberg, West Germany, mixed 1:1 with peat) in a greenhouse with a photoperiod of *ca* 16 hr at 20–22° in the dark and 25–28° in the light.

*Substrates.* Coenzyme A (free acid) was purchased from Serva (Heidelberg, West Germany). 3-Caffeoylquinate (chlorogenic acid) came from Fluka (Neu-Ulm, West Germany). *p*-Coumaroyl- and caffeoyl-CoA were gifts from A. Peters (this institute). Other substrates were extracted from the following plants: *p*-coumaroyl-, caffeoyl- and feruloyltartronate from primary leaves of mung bean [1]; 2-acetyl-3-(*p*-coumaroyl)-tartrate from cotyledons of *Spinacia oleracea* [18; Strack *et al.*, unpublished]; *p*-coumaroylmalate from leaves of *Raphanus sativus* [6]; 1-(*p*-coumaroyl)-glucose from petals of *Antirrhinum majus* [19]. *p*-Coumaroyltartrate was prepared hydrolytically from 2-acetyl-3-(*p*-coumaroyl)-tartrate [Strack *et al.*, unpublished]. Principal methods of extraction and procedures of chromatography were published elsewhere [20].

*Partial purification of transferase.* Four hundred 4-day-old primary leaves were homogenized in 200 ml Tris–HCl buffer (100 mM, pH 8.0) containing 10 mM DTE (dithioerythritol) and 1 mM EDTA and the transferase extracted as described below. The $(NH_4)_2SO_4$-precipitated (30–80% satn) protein was redissolved in 5 ml Tris–HCl buffer and this was dialysed against the same buffer containing 20 mM 2-mercaptoethanol. The protein soln was applied to a column of DEAE-cellulose (DE-52, 10 × 1.5 cm). The column was washed with 60 ml Tris–HCl buffer (100 mM, pH 8.0, 20 mM 2-mercaptoethanol) before the following linear gradient was applied: with 120 ml from 0 to 0.5 M KCl in Tris–HCl buffer. Three-ml fractions were collected. Flow rate was 1 ml/min. Highest activity was found in fraction 12. This protein was used for the determination of some properties of the enzyme.

*Standard enzyme preparation. Transferase*: Primary leaves and other organs from 20 plants were ground (15 min) in a precooled (4°) mortar in the presence of insoluble Polyclar AT, Dowex 1X8 ($Cl^-$) (2.5 g/10 ml buffer), quartz sand and 25 ml Tris–HCl buffer (100 mM, pH 8.0) containing 20 mM DTE and 1 mM EDTA [3–5]. Preparation including CoA ligase was done with 100 mM KPi (pH 7.0) in the presence of insoluble Polyclar AT and 2 mM DTE. *Hydroxylase*: Primary leaves and other organs from 25 plants were ground (15 min) in a precooled (4°) mortar in the presence of insoluble Polyclar AT, quartz sand and NaPi–citric acid buffer (100 mM/50 mM, pH 5.3).

The homogenates from both enzyme preparations (transferase or hydroxylase) were passed through Miracloth and the filtrates centrifuged at 48 000 *g* for 20 min. Enzyme activities were prepared from 30–80% $(NH_4)_2SO_4$ satn. The precipitated protein was filtered through Sephadex G-25 (Pharmacia PD-10 columns). The eluates were used as source of enzymatic activities. Protein contents were determined by the method of ref. [21] using bovine serum albumin as standard.

*Standard enzyme assay. Transferase forward reaction*: The reaction mixture contained in a total vol. of 250 μl 40 mM KPi (pH 7.0), 10 mM DTE, 1 mM EDTA, 0.2 mM *p*-coumaroyl-CoA, 0.4 mM tartronic acid and 25 μl protein soln. The reaction was started by the introduction of tartronic acid. Enzyme assays including activity of CoA ligase contained the following components: 50 mM KPi (pH 7.0), 1 mM DTE, 1 mM $MgCl_2$, 1 mM ATP, 0.2 mM CoA, 2 mM hydroxycinnamic acid and 5 mM tartronic acid. *Transferase reverse reaction*: The mixture contained in a total vol. of 250 μl 40 mM KPi (pH 7.0), 10 mM DTE, 1 mM EDTA, 0.2 mM hydroxycinnamoyltartronate, 0.4 mM CoA and 25 μl protein soln. The reaction was started by the introduction of CoA. *Hydroxylase*: The mixture contained in a total vol. of 170 μl 0.2 mM *p*-coumaroyltartronate, 0.5 M $(NH_4)_2SO_4$, 2.4 mM ascorbic acid, 3.5 mM EDTA in 0.07 M NaPi–0.03 M citric acid buffer (pH 7.0) [7, 8]. The reaction was started by the introduction of 15 μl protein soln. Both transferase and hydroxylase activities were stopped after incubation at 30° for 10 min by transferring the mixtures to a freezer (−20°) or by immediate HPLC analyses.

*Determination of enzyme activities.* Transferase activity (forward and back reaction) was either photometrically measured by a method developed by Zenk and co-workers [22, 23] or by means of HPLC as described in this paper (Fig. 2). The latter was also used for determination of the hydroxylase activity. Caffeic acid and caffeoyltartronate as products from hydroxylase activities were co-chromatographically (HPLC and TLC as described below) identified using the following standard compounds: caffeic acid purchased from Serva (Heidelberg, West Germany) and caffeoyltartronate isolated from mung bean primary leaves [1]. *p*-Coumaroyl- and caffeoyl-CoA as products from transferase activities were co-chromatographically identified using synthetic CoA thioesters. Feruloyl-CoA was tentatively identified by its characteristic behaviour in HPLC relative to that of the applied feruloyltartronate. Data from studies on development and organ localization of the enzyme were proved by mixing low and high enzymatic activities and strict linearities were found.

*pH Optimum.* The following buffer systems (0.1 M each) were used to determine the pH dependence of the enzymatic activities: MES, 2-(*N*-morpholino)ethanesulphonic acid (pH 5.0–7.0); KPi (pH 5.5–8.0); TRICINE, *N*-tris(hydroxymethyl)methylglycine (pH 7.5–9.5).

*Kinetic properties.* Apparent $K_m$ and $V_{max}$ values were graphically estimated according to ref. [24].

*Treatment with tentoxin.* Seeds were imbided (12 hr) with an 80 μM aq. soln of tentoxin (Sigma, München, West Germany) according to ref. [15] and subsequently 5-day-old plants were grown in a defined soil (see above) under greenhouse conditions. Control plants were imbibed with $H_2O$ alone and one set of plants grown under identical conditions as the tentoxin treated ones and a second set under continuous dark.

*Chromatography.* TLC of the hydroxycinnamoyltartronates and -malates was done as described previously [1, 6] and on silica

gel in EtOAc–HOAc–$HCO_2H$–$H_2O$ (100:11:11:27) [25] (*p*-coumaroyltartronate: $R_f$ 0.49).

*HPLC*: The liquid chromatograph used was from LKB Instrument GmbH (Gräfelfing, West Germany) and included the following: HPLC pump (LKB 2150), HPLC controller (LKB 2152), UV/Vis variable wavelength monitor (LKB 2151), solvent conditioner (LKB 2156). Injection was done via a Rheodyne rotary valve (Rheodyne Inc., Cotati, CA, USA) with a 20 μl loop. The chromatographic column used was prepared with Shandon ODS Hypersil 5 μm (Bischoff Analysentechnik und -geräte GmbH, Leonberg, West Germany). Quantitative calculations were obtained with a Shimadzu Data Processor Chromatopac C-R3A (Shimadzu Corporation, Kyoto, Japan) using authentic *p*-coumaroyltartronate [1] and *p*-coumaroyl-CoA as standards. For other details see Fig. 2 and ref. [1].

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