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# Partial Purification and Characterization of Hydroxycinnamoyl-Coenzyme A:Tyramine Hydroxycinnamoyltransferase from Cell Suspension Cultures of *Solanum tuberosum*[1]

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**A pathogen elicitor-inducible soluble acyltransferase (tyramine hydroxycinnamoyltransferase [THT], EC 2.3.1), which catalyzes the transfer of hydroxycinnamic acids from hydroxycinnamoyl-coenzyme A (CoA) esters to tyramine in the formation of *N*-hydroxycinnamoyltyramine, was partially purified with a 380-fold enrichment and a 6% recovery from cell-suspension cultures of potato (*Solanum tuberosum* L. cv Datura). The enzyme showed specific activities of 33 mkat (kg protein)$^{-1}$ (formation of feruloyltyramine). The apparent native $M_r$ was found to be approximately 49,000. Highest activity was at pH 6.8 in K-phosphate. The isoelectric point of the enzyme was approximately pH 5.2. The apparent energy of activation was calculated to be 96 kJ mol$^{-1}$. The enzyme activity was stimulated more than 5-fold by 10 mM $Ca^{2+}$ or $Mg^{2+}$. The apparent $K_m$ values were 36 μM for feruloyl-CoA and 85 and 140 μM for cinnamoyl- and 4-coumaroyl-CoA, respectively. The $K_m$ value for tyramine in the presence of feruloyl-CoA was 22 μM. In the presence of 4-coumaroyl-CoA, however, the $K_m$ for tyramine increased to about 230 μM. The mode of action was an iso-ordered bi bi mechanism in which A, B, P, and Q equal hydroxycinnamoyl-CoA, tyramine, *N*-hydroxycinnamoyltyramine, and CoA, respectively. Thus, the reaction occurred in a ternary complex of the enzyme and substrates. The equilibrium constant of the reaction was determined to be 1.3 × 10$^4$. This gave a $\Delta G^{o\prime}_{eq}$ value of −23.5 kJ mol$^{-1}$.**

Amides of hydroxycinnamic acid formed with tyramine and various other amines, which accumulate as soluble constituents, are widespread in higher plants (Martin-Tanguy et al., 1978). There is no definite conclusion yet about their functions, although their possible role in plant growth processes or antimicrobial and antiviral effects have been discussed (Martin-Tanguy, 1985; Martin-Tanguy and Negrel, 1987). Their proposed roles in developmental processes, such as in the tuberization process of potato and in the flowering process of solanaceous species, are doubtful (Leubner-Metzger and Amrhein, 1993).

In recent years, hydroxycinnamic acid tyramine amides have repeatedly been found also as insoluble constituents of cell-wall fractions (Clarke, 1982). These amides may constitute a preformed, or induced, barrier against pathogens, along with various other cell-wall phenolics (Matern and Kneusel, 1988; Graham and Graham, 1991; Beimen et al., 1992). They may affect the digestibility of cell walls (Fry, 1986) but may also directly inhibit hyphal growth of a potential fungal invader (Grandmaison et al., 1993).

So far as is known, hydroxycinnamic acid amide formation requires activation of the hydroxycinnamic carboxylic group by way of its CoA thioester (Strack and Mock, 1993). The enzyme responsible for the formation of amides of hydroxycinnamic acid with tyramine, THT (EC 2.3.1), was described first in *Nicotiana tabacum* leaves infected by tobacco mosaic virus (EC 2.3.1.110; Negrel and Martin, 1984). It was also detected in *Nicotiana glutinosa* and *Eschscholtzia californica* cell-suspension cultures, following elicitation by chitosan (Villegas and Brodelius, 1990), and in *Solanum tuberosum* tuber discs induced by wounding (Negrel et al., 1993).

THT appears to be also involved in the resistance reactions of potato against pathogen attack (Hahlbrock and Scheel, 1989). It catalyzes the formation of cell-wall-bound *N*-feruloyl- and *N*-4-coumaroyltyramine (Clarke, 1982; Friend, 1985). As part of our studies of the induction of THT synthesis by as yet unknown components from *Phytophthora infestans* and control of the THT gene in potato, we report here the partial purification and detailed characterization of THT from potato cell cultures.

## MATERIALS AND METHODS

### Plant Material and Elicitor Preparation

Cell-suspension cultures from potato (*Solanum tuberosum* L. cv Datura) were maintained on Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with either 1 mg 2,4-D L$^{-1}$ or 2 mg 2,4-D L$^{-1}$ and B5 vitamins according to Gamborg et al. (1968). Cultivation conditions were as described by Kombrink and Hahlbrock (1986).

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Abbreviations: FPLC, fast protein liquid chromatography; $R_t$, retention time; THT, hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase; *V*, maximal velocity.

The cultures were supplied with elicitor preparations from *Phytophthora infestans* to increase enzyme activity. Briefly, concentrated culture filtrate was prepared from *P. infestans* race 4 grown for 5 weeks in liquid Henniger medium (Henniger, 1959) as described previously (Rohwer et al., 1987). Filter-sterilized elicitor solution was added to suspension cultures 7 d after transfer to give a final concentration of 10 $\mu$g mL$^{-1}$ Glc equivalents as determined by the method of Dubois et al. (1956).

## Chemicals and Substrates

With the exception of Fractogel TSK Butyl-650 (M), which came from Merck (Darmstadt, Germany), all of the other column materials for enzyme purification were obtained from Pharmacia LKB (Freiburg, Germany). Various phenolic compounds and amines were purchased from Merck, Germany), Sigma (München, Germany), and Serva (Heidelberg, Germany). Hydroxycinnamoyl-CoA and vanilloyl-CoA esters were chemically synthesized by the ester-exchange reaction via the acyl *N*-hydroxysuccinimide esters (Stöckigt and Zenk, 1975) and purified on polyamide (Macherey-Nagel, Düren, Germany) columns (Strack et al., 1987). *N*-Feruloyl- and *N*-4-coumaroyltyramine, which were prepared by the above ester-exchange reaction, were provided by H. Keller (Köln, Germany). Adjustment of molarities was done by applying known extinction coefficients in the case of the cinnamoyl- and hydroxycinnamoyl-CoA esters (Gross and Zenk, 1966; Stöckigt and Zenk, 1975; Hrazdina et al., 1976). Vanilloyl-CoA was quantified by comparison (HPLC) with vanillic acid liberated from the CoA ester by alkaline treatment (30 min in 0.1 N NaOH at room temperature). The tyramine amides were quantified by comparison (HPLC at 280 nm) with authentic free hydroxycinnamic acids corrected for the *A* of tyramine.

## THT Purification

Purification steps 1, 2, and 3 were carried out at 0 to 4°C and the FPLC steps 4 and 5 were done at room temperature. Protein was determined by the Bradford method (Bradford, 1976) using BSA as the standard.

### *Step 1: Preparation of Crude Extract*

Frozen cells (100 g) were disrupted with an Ultra Turrax homogenizer in the presence of 200 mL of 50 mM Tris-HCl (pH 7.5), 5 mM DTT (buffer A), and 20 g of Polyclar AT. The homogenate was stirred for 20 min and then centrifuged for 20 min at 25,000*g*.

### *Step 2: Ammonium Sulfate Treatment*

Solid $(NH_4)_2SO_4$ was added to the protein extract to 35% saturation. This solution was stirred for 30 min and then centrifuged for 20 min at 25,000*g* to remove precipitated protein.

### *Step 3: Hydrophobic Interaction Chromatography on TSK Butyl-650 (M)*

The protein solution from step 2 was chromatographed on a TSK Butyl-650 (M) column (22.5 × 2.6 cm i.d.) at a flow rate of 1.25 mL min$^{-1}$. The column was equilibrated with 35% saturated $(NH_4)_2SO_4$ in buffer A (buffer B) before the following gradient was applied: buffer B to buffer A with a total volume of 1100 mL. Active protein was concentrated by ultrafiltration (Diaflo PM30, Amicon, Beverly, MA).

### *Step 4: Ion Exchange on FPLC-Mono Q*

Active protein obtained from step 3 was applied to a FPLC-Mono Q column (5 × 0.5 cm i.d.). The column was equilibrated with buffer A before the following linear gradient at a flow rate of 1 mL min$^{-1}$ was applied: buffer A to 1 M KCl in buffer A (buffer C); buffer A to 50% buffer C (in buffer A plus buffer C) with a volume of 30 mL, followed by 50% buffer C to 100% buffer C with a volume of 15 mL. Active protein was concentrated by ultrafiltration.

### *Step 5: Molecular Exclusion on FPLC-Superdex TM-75*

Active protein obtained from step 4 was applied to a FPLC-Superdex TM-75 column (60 × 1.6 cm i.d.). Protein was eluted with 0.1 M KCl in buffer A at a flow rate of 1 mL min$^{-1}$.

## HPLC

### *System 1*

The liquid chromatograph (two-pump system; Pharmacia-LKB) was equipped with a 5-$\mu$m Nucleosil $C_{18}$ column (25 cm × 4 mm i.d.; Macherey-Nagel), and the following solvents were 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-$\mu$L loop. Compounds were detected photometrically at 260, 280, and 320 nm (LKB UV/Vis variable wavelength monitor) and quantified by external standardization with authentic feruloyltyramine using a Shimadzu Data Processor Chromatopac C-R3A (Shimadzu Corp., Kyoto, Japan).

### *System 2*

The liquid chromatograph (Waters 600-MS System; Millipore, Eschborn, Germany) was equipped with the same column as in system 1. The following solvents were used: solvent A = 1.5% phosphoric acid in water; solvent B = 80% acetonitrile in water. Injections of 20 $\mu$L were carried out by an automatic sampler (Waters 717 autosampler). Compounds were detected photometrically at the wavelengths as in system 1 by a photodiode array detector (Waters 996 photodiode array detector) and quantified as in system 1 using the Millenium software 2010 (Millipore).

### Determination of Enzyme Activities

*Forward Reaction*

The reaction mixture contained in a total volume of 30 μL 250 mM K-phosphate (pH 6.8), 0.5 mM feruloyl-CoA, 20 mM tyramine, and 10 μL of protein solution. The reaction (at 30°C) was started by the addition of protein and stopped after 2 to 30 min by the addition of 30 μL of 10% TCA and transferring the mixture into liquid nitrogen. A more sensitive assay was used for activity determinations during enzyme purification steps by using 1 M imidazole-HCl (pH 6.5) instead of K-phosphate. If not stated otherwise, THT activity represents the formation of feruloyltyramine.

The products formed during the incubations were determined by HPLC as follows. System 1 with Pharmacia-LKB equipment: at a flow rate of 1 mL $min^{-1}$ linear gradient elution from 60% solvent B in (A plus B) within 6 min to 100% B in (A plus B), followed by 5 min at 100% B ($R_t$ of feruloyltyramine = 7.9 min); system 2 with Millipore-Waters equipment: at a flow rate of 1 mL $min^{-1}$ linear gradient elution fom 40% solvent B in (A plus B) within 9 min to 70% B in (A and B) followed by 4 min at 100% B ($R_t$ of feruloyltyramine = 6.5 min). The gradient used in the inhibition studies was the same as for determination of the reverse reactions ($R_t$ of 4-coumaroyltyramine = 10.9 min; $R_t$ of feruloyltyramine = 11.3 min).

*Reverse Reaction*

The assay mixture contained, in a total volume of 30 μL, 500 mM Tris-HCl (pH 7.2), 20 mM CoA, 4 mM feruloyltyramine, and 10 μL of protein solution. The reaction (at 30°C) was started by the addition of protein and stopped (see above) after 1 and up to 8 h. The formation of feruloyl-CoA was monitored using the Millipore-Waters HPLC system: at a flow rate of 1 mL $min^{-1}$ linear gradient elution from 30% solvent B in (A plus B) within 12 min to 60% B in (A plus B) followed by 4 min at 100% B ($R_t$ feruloyl-CoA = 4.4 min).

### Determination of the *K*

The *K* of the THT-catalyzed reaction was determined by estimation of the four reaction components from assays running the reverse reaction. The components were separated and quantified by using the Waters HPLC system: at a flow rate of 1 mL $min^{-1}$ linear gradient elution from solvent A to 35% solvent B in (A plus B) within 20 min and then to 70% B within 10 min followed by 4 min at 100% B ($R_t$ of tyramine = 8.5 min; $R_t$ of CoA = 13.8 min; $R_t$ of feruloyl-CoA = 24.0 min; $R_t$ of feruloyltyramine = 28.7 min).

### Determination of the Energy of Activation

Initial velocities of THT-catalyzed reactions were determined at different temperatures (0–68°C). The apparent energy of activation was determined from the linear low-temperature section of the Arrhenius plot.

### Enzyme Characterization

*$M_r$ Determination*

The native $M_r$ was determined on an FPLC-Superdex TM-75 column (see above). The following reference proteins (Serva) were used: Cyt *c* ($M_r$ 12,400), equine myoglobin ($M_r$ 17,800), egg albumin ($M_r$ 45,000), BSA ($M_r$ 67,000), and rabbit aldolase ($M_r$ 160,000). $K_{av}$ on Superdex column = $(V_e - V_o) \times (V_t - V_o)$ was estimated using Blue Dextran 2000 ($V_o$) (Pharmacia-LKB) and dinitrophenylalanine ($V_t$) (Serva).

*pH Optimum*

The following buffer systems at 150 mM each were used to determine the pH optimum of THT activity: Na-citrate (pH 4.5–6.3), K-Mes (pH 5.2–7.0), imidazole-HCl (pH 5.9–7.7), K-phosphate (pH 6.1–7.6), Tris-HCl (pH 7.1–8.7), and Gly (pH 8.1–9.9). The actual pH values were measured after the reaction assays were mixed.

*IEF*

Determination of the pI was carried out on "Servalyt precotes" (pH 4–7; 125 × 125 mm; Serva) at 3 W. After prefocusing (prescription by Serva), we loaded 10 μL of enzyme solution onto the gel (55 mm from the anodic and 70 mm from the cathodic edge). Electrophoresis was started at 500 V and terminated after 3 h at 1700 V.

*Kinetic Properties*

For kinetic measurements the 380-fold purified enzyme was used. Apparent $K_m$ and *V* values at fixed concentrations of the respective second substrate were obtained by Lineweaver-Burk (Lineweaver and Burk, 1934) or Hanes plots (Hanes, 1932). The kinetics data were calculated from regression analyses. In the product-inhibition experiments, the effects of the presence of five fixed concentrations each of free CoA and 4-coumaroyltyramine on the kinetics constants toward feruloyl-CoA and tyramine were studied. The product-inhibition patterns were obtained from Hanes plots.

## RESULTS AND DISCUSSION

### Enzyme Purification

THT from potato cell cultures was isolated as a soluble enzyme 5 d after elicitation with a culture filtrate from *P. infestans*. The enzyme was purified 380-fold with a yield of 6% according to the procedure listed in Table I. The final specific activity obtained was 33 mkat (kg protein)$^{-1}$ in the formation of *N*-feruloyltyramine (standard assay). Hydrophobic interaction chromatography was the decisive step in the successful purification sequence. Figure 1 illustrates the results of chromatography on the hydrophobic Fractogel TSK Butyl column.

**Table I.** *Purification of THT from potato cell cultures*

| Purification Step | Protein | Total Activity | Specific Activity | Yield | Purification |
|---|---|---|---|---|---|
| | *mg* | *nkat* | *mkat kg$^{-1}$* | % | *fold* |
| Crude extract | 435 | 38 | 0.087 | 100 | 1 |
| $(NH_4)_2SO_4$ (35%) supernatant | 346 | 35 | 0.1 | 91 | 1.2 |
| Fractogel TSK Butyl-650 (M)[a] | 1.12 | 8.0 | 7.1 | 21 | 82 |
| FPLC-Mono Q[a] | 0.40 | 4.6 | 11.6 | 12 | 133 |
| FPLC-Superdex TM-75 | 0.069 | 2.3 | 33.4 | 6.0 | 384 |

[a] Active protein was concentrated by ultrafiltration.

## Stability

Both crude protein preparations and the partially purified enzyme could be stored at −80°C for several months without apparent loss of activity. However, both types of preparation lost about 50% of enzyme activity within 6 months when stored at −20°C. At 4°C a loss of 25% of enzyme activity was observed after 24 h. This could be prevented by the addition of an SH-group reagent (e.g. 5 mM DTT or 10 mM 2-mercaptoethanol). Dilution of the purified enzyme with extraction buffer resulted in a dramatic irreversible loss of activity. A 4-fold dilution gave 50%, and a 10-fold dilution gave 95% loss.

## General Properties

The formation of feruloyl- and 4-coumaroyltyramine was positively correlated with protein and was linear with time up to 50 min with 92 μg protein (mL assay)$^{-1}$ from the standard crude protein preparation and up to 20 min with 0.36 μg of partially purified enzyme. Figure 2 shows HPLC diagrams from a standard THT assay, including UV spectra of the substrate feruloyl-CoA and the product feruloyltyramine, obtained by a photodiode array detector. No product was formed after heating the assay mixture for 5 min at approximately 100°C.

The apparent native $M_r$ of THT was determined to be about 49,000, based on calibrated FPLC-Superdex TM-75 chromatography. This is in the range of the $M_r$ estimated for THT from tobacco leaves (Negrel and Martin, 1984).

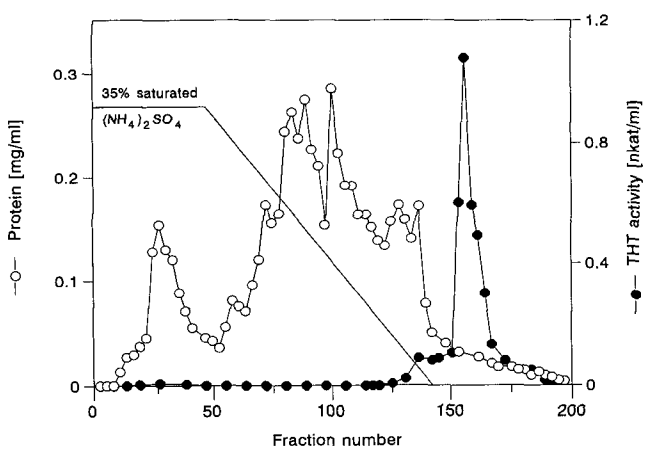



**Figure 1.** Elution profiles of protein and THT activity from a $(NH_4)_2SO_4$ gradient (35% saturation to 0) on TSK Butyl-650 (M).

The effect of pH on the catalytic efficiency of THT was tested from pH 4.5 to 9.9. There were some differences in pH optima, depending on the buffer used. The optima found were at pH 6.6 in K-Mes, 6.8 in K-Pi, and 6.5 in imidazole-HCl, with 50% of the maximal activity in K-Mes at approximately pH 6.0 and in Gly at approximately 9.1. IEF showed a single band of enzyme activity with a pI of approximately pH 5.2.

The effect of temperature on enzyme activity was tested from 0 to 68°C. The maximal rate of product formation was found at approximately 55°C. From these data the apparent energy of activation was calculated from a linear Arrhenius plot to be 96 kJ mol$^{-1}$.

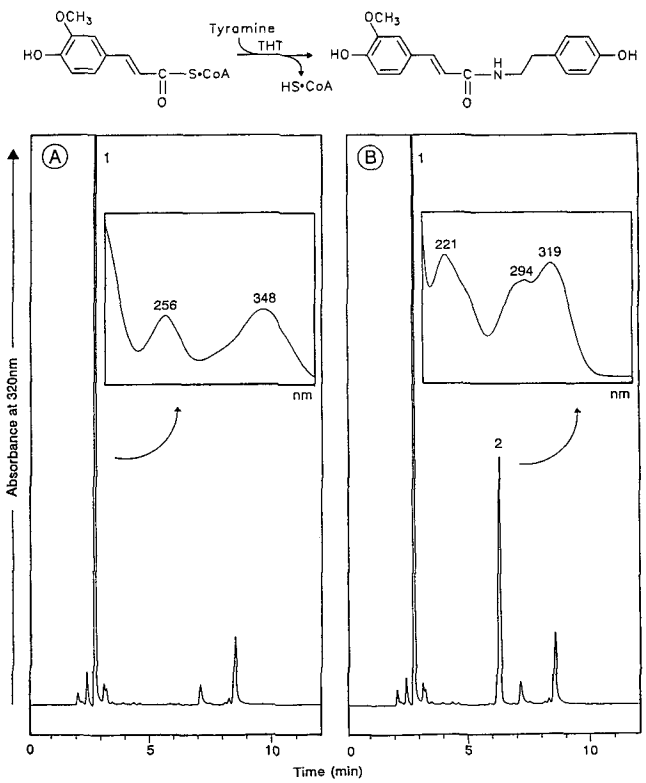



**Figure 2.** Reaction scheme of THT activity and its HPLC analysis (Waters HPLC system; 20-μL injection volume) immediately after mixing the standard assay (A) and after a 20-min reaction time (B); 0.2 *A* unit full scale. Insets show the UV spectra obtained from a photodiode array detector of the acyl donor feruloyl-CoA (peak 1) and the product *N*-feruloyltyramine (peak 2 = 105 pmol).

THT activity was positively affected by divalent cations. The enzyme activity was stimulated in a saturating mode more than 5-fold in the presence of 10 mM $Ca^{2+}$ ($CaCl_2$) or 10 mM $Mg^{2+}$ ($MgCl_2$). $Mn^{2+}$ ($MnSO_4$) and $Cu^{2+}$ ($CuSO_4$) showed a 3-fold stimulation at 1.5 and 0.5 mM, respectively, but were strongly inhibitory at higher concentrations. $Fe^{2+}$ ($FeSO_4$) and $Zn^{2+}$ ($ZnSO_4$) inhibited the activity 50%, at about 0.3 and 0.4 mM, respectively.

### Substrate Specificity and Kinetics

The substrate specificity of the THT activity tested with various phenolic CoA esters and amines as possible donors and acceptors, respectively, is summarized in Table II. The enzyme had a high affinity toward feruloyl-CoA (apparent $K_m$ 36 μM) as donor, followed by cinnamoyl-CoA (85 μM). However, the latter gave highest specificity ($V/K_m$), followed by feruloyl-CoA (46%) and 4-coumaroyl-CoA (32%). This is consistent with the behavior of the THT from tobacco leaves (Negrel and Martin, 1984), which, however, did not accept caffeoyl-CoA. In contrast, this CoA ester was converted to caffeoyltyramine by the THT from potato cell cultures, with a specificity of 11% compared to cinnamoyl-CoA. There was weak activity with vanilloyl-CoA (same aromatic substitution pattern as ferulic acid) for which the $K_m$ of 2400 μM and a relative $V/K_m$ value of 0.5% were obtained. Obviously, the propenoic moiety of the hydroxycinnamic acids is an important determinant of acceptor specificity.

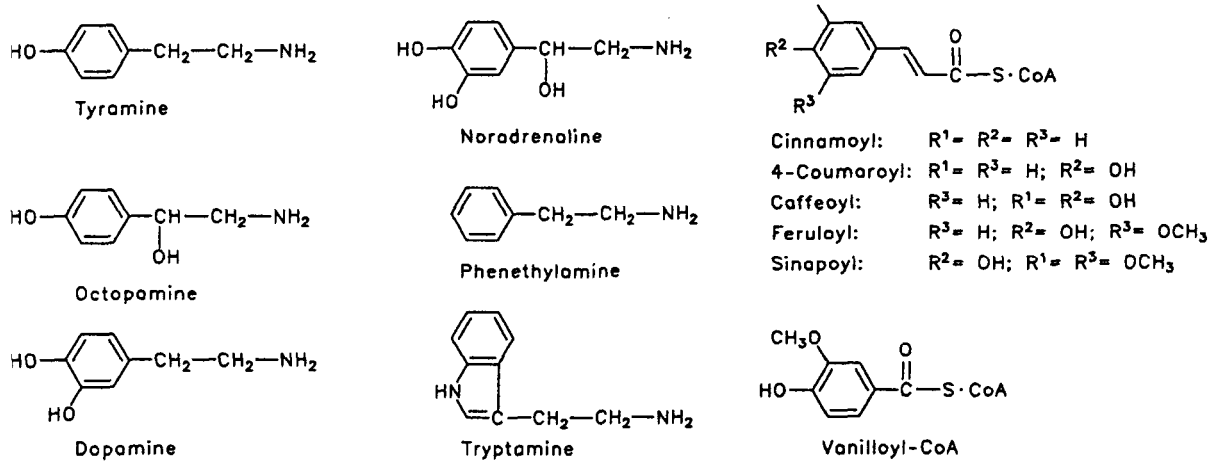
There was a pronounced specificity ($V/K_m$) and affinity ($K_m$) for tyramine as acceptor ($K_m$ 22 μM in the presence of feruloyl-CoA), followed by octopamine with a $K_m$ of 40 μM and specificity of about 50% compared to tyramine. The $K_m$ for tyramine in the presence of 4-coumaroyl-CoA was found to be 10 times higher (232 μM). Other aromatic amines tested were poor acceptors. The decrease of affinities toward the acceptors depends mostly on variations of the aromatic substitution patterns (compare tyramine, dopamine, and phenethylamine). In case of a completely different ring system (see tryptamine), both the $K_m$ and $V$

**Table II.** *Substrate specificity of THT activity*

| Substrate | $K_m$ | $V$ | $V/K_m$ |
|---|---|---|---|
| | mM | % | kat × (M kg)$^{-1}$ |
| Donors | | | |
| Cinnamoyl-CoA | 0.085 | 100[a] | 1080 |
| Feruloyl-CoA | 0.036 (0.033)[b] | 20 | 500 |
| 4-Coumaroyl-CoA | 0.14 | 54 | 350 |
| Caffeoyl-CoA | 0.23 | 43 | 122 |
| Sinapoyl-CoA | 0.20 | 24 | 110 |
| Vanilloyl-CoA | 2.40 | 15 | 6 |
| Acceptors[c] | | | |
| Tyramine[d] | 0.022 (0.022)[b] | 51 | 1000 |
| Octopamine | 0.040 | 53 | 575 |
| Dopamine | 0.36 | 77 | 92 |
| Noradrenaline | 1.6 | 100[e] | 27 |
| Phenethylamine | 2.0 | 23 | 5.0 |
| Tryptamine | 26 | 4 | 0.07 |

[a] 92 mkat × (kg protein)$^{-1}$. [b] Calculated from the inhibition patterns (Fig. 3). [c] With feruloyl-CoA as acyl donor. [d] $K_m$ value was determined to be 10 times greater (232 μM) in the presence of 4-coumaroyl-CoA as acyl donor. [e] 43 mkat × (kg protein)$^{-1}$.

Structures of substrates:



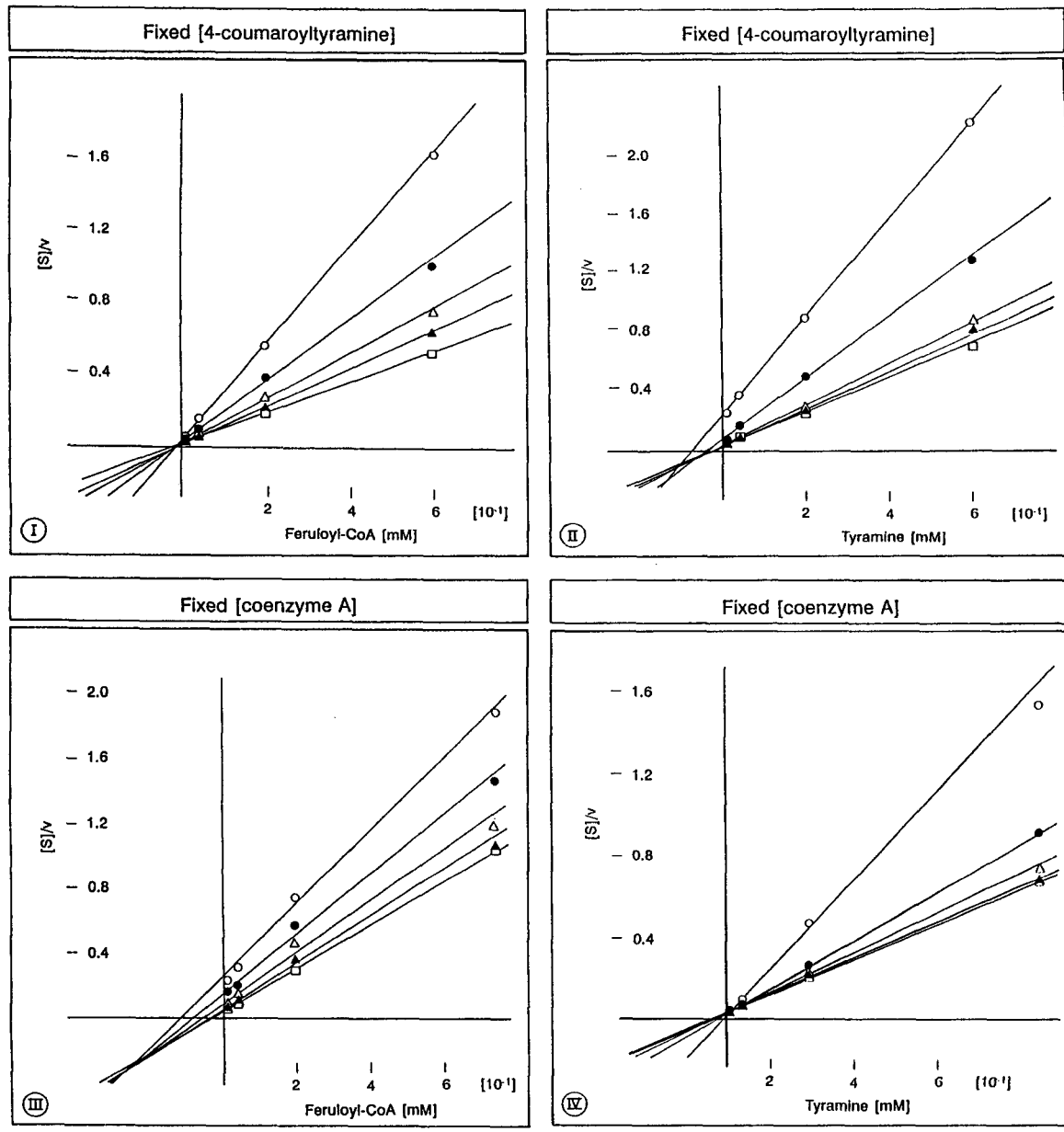



**Figure 3.** Hanes plots of THT activity with feruloyl-CoA or tyramine saturated with the respective second substrate (10 mM tyramine or 2 mM feruloyl-CoA) in the absence or presence of different concentrations of 4-coumaroyltyramine (0.075, 0.2, 0.75, and 2.0 mM) or free CoA (0.4, 1.0, 4.0, and 10 mM) ($v$ = mkat × [kg protein]$^{-1}$). 4-Coumaroyltyramine was used as the inhibiting product to get exact quantitations of low concentrations of the enzymatically formed feruloyltyramine by HPLC analysis (separation of the two amides with $\Delta R_t$ of 0.4 min). The patterns of intersecting lines in I and IV are consistent with uncompetitive and in II and III with noncompetitive product inhibitions.

values are dramatically affected. Tyr and agmatine, as well as the polyamines putrescine, cadaverin, spermidine, and spermine, were not accepted. The fact that THT activity showed only one isoelectric species, and the observation that the different substrates acted as competitive inhibitors when added as a second donor or acceptor, indicates that the enzyme activity is most likely associated with a single protein. For example, feruloyltyramine formation at 50 μM feruloyl-CoA was reduced to 50% in the presence of 130 μM

**Table III.** *Product-inhibition pattern of THT activity saturated with the respective second substrate (see also Fig. 3)*

| Product | Substrate | |
|---|---|---|
| | Feruloyl-CoA (A) | Tyramine (B) |
| 4-Coumaroyltyramine (P) | Uncompetitive[a] | Noncompetitive[b] |
| Coenzyme A (Q) | Noncompetitive | Uncompetitive |

[a] Intersection on $y$ axis. [b] Intersection below the $x$ axis in Hanes plot of product-inhibition kinetics of THT activity.

cinnamoyl-CoA and cinnamoyltyramine formation at 50 μM cinnamoyl-CoA was reduced to 50% in the presence of 340 μM feruloyl-CoA. In contrast to the potato system, several THT isoforms have been found in tobacco mosaic virus-inoculated tobacco leaves (Fleurence and Negrel, 1989).

Lineweaver-Burk plots of initial velocities with tyramine or the hydroxycinnamoyl-CoA at different fixed levels of the products gave families of straight lines with intersections above the $x$ axis (not documented). The dissociation constant for feruloyl-CoA was found to be greater than the respective Michaelis constant ($K_i$ = 80 μM, $K_m$ = 36 μM). This is in accordance with a sequential bi bi mechanism. To gain more information about this mechanism, the product-inhibition pattern of enzyme activity was determined. In these measurements, one substrate was kept constant at saturating conditions, the other substrate was varied, and one of the two products was present as inhibitor at four different fixed concentrations. Four Hanes plots were obtained (Fig. 3). The product-inhibition mechanisms derived from these plots are listed in Table III. The $K_i$ values for 4-coumaroyltyramine and CoA were calculated to be 95 μM and 2.1 mM, respectively. According to Segel (1975), this inhibition pattern is in agreement with an iso-ordered bi bi mechanism (no product is released before the formation of the ternary complex between the enzyme and the two substrates) in which, according to the Cleland nomenclature, A, B, P, and Q equal hydroxycinnamoyl-CoA, tyramine, *N*-hydroxycinnamoyl-tyramine, and CoA, respectively. It is assumed that the enzyme undergoes a conformational change during catalysis, in which the substrates and the products bind to different protein conformations. The assumption that the CoA ester is the first substrate to bind to the enzyme is supported by the different $K_m$ values obtained for tyramine in the presence of different hydroxycinnamoyl-CoAs, as shown for feruloyl- and 4-coumaroyl-CoA (see above). This might indicate that the enzyme isomerizes after binding the first substrate, but this will require more detailed kinetic studies.

### Reversibility and Energetics of the Reaction

In agreement with the postulated reaction mechanism, the tyramine amide formation is freely reversible. When feruloyl- or 4-coumaroyltyramine were assayed in the presence of free CoA, feruloyl- or 4-coumaroyl-CoA and free tyramine were formed. The reverse reactions were allowed to proceed to equilibrium (incubation time 6–8 h) and the equilibrium concentrations of all reactants were calculated by HPLC analysis. During the incubation period, active protein was added to assure enzyme activity. The $K$ was calculated from the means of concentrations (mM) derived from five independent experiments. The equation reads $K = ([\text{CoA}] \times [\text{feruloyltyramine}]) \times ([\text{feruloyl-CoA}] \times [\text{tyramine}])^{-1} = (8.3 \times 0.4) \times (0.01514 \times 0.0168)^{-1} = 1.3 \times 10^4$. This gave a $\Delta G^{\circ\prime}_{eq}$ value ($-R \times T \times \ln K$) = −23.5 kJ $mol^{-1}$. This relatively high negative value is to be expected for CoA-dependent transferase reactions.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