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PARTIAL PURIFICATION OF TYRAMINE FERULOYL TRANSFERASE FROM TMV INOCULATED TOBACCO LEAVES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; enzyme purification; tyramine feruloyl transferase; isoenzymes.

Abstract—After extraction from *Nicotiana tabacum* cv Xanthi n.c. leaves inoculated with tobacco mosaic virus, tyramine feruloyl transferase was purified 46-fold with a 24% yield by ammonium sulphate fractionation followed by DEAE cellulose and hydroxylapatite chromatography. The partially purified enzyme can use four cinnamoyl-CoA derivatives and seven tyramine analogues as substrates. Three fractions with different specificities towards various cinnamoyl-CoA derivatives and toward tyramine and dopamine can be separated by ion exchange chromatography on DEAE Trisacryl. These three fractions may contain several TFT isoforms.

INTRODUCTION

Extracts of *Nicotiana tabacum* cv Xanthi n.c. leaves previously inoculated with tobacco mosaic virus (TMV) are known to catalyse the synthesis of cinnamic acid amides of tyramine and tyramine analogues (dopamine etc.). More than 20 different amides can be obtained *in vitro* using the crude enzymic extract [1]. *In vivo* only three amides (cinnamoyl-, *p*-coumaroyl- and feruloyltyramine) have been characterized so far [2]. The fate of these amides in tobacco leaves has not been completely elucidated although several lines of evidence indicate that they could be incorporated into cell walls [2–4]. The purification of TFT, allowing its histological and cytological localization by immunological means, could be useful to confirm the involvement of the amides in the formation of lignin-like compounds. It also has a theoretical interest as only one amine cinnamoyl transferase (ACT from barley seedlings) has been purified so far [5], although this family of enzymes is involved in several important aspects of plant metabolism [1, 5, 6].

We describe here the partial purification of TFT and a procedure to split the initial crude extract into three active fractions exhibiting different specificities towards both cinnamoyl-CoA derivatives and tyramine analogues.

RESULTS

Stability of TFT in the presence of thiols

TFT activity extracted from tobacco leaves is stable providing a thiol is added to the extraction buffer [1]. Dithiothreitol (DTT) is more effective to protect the activity in the extract obtained after ammonium sulphate concentration than mercaptoethanol (ME) (25% loss after incubation for 24 hr at 4° with 10 mM ME, 10% with 5 mM DTT). However addition of DTT in the buffers used to elute proteins from chromatography columns results in a rapid loss of activity. We have tested the effect of several sulphhydryl reagents on enzymic extracts obtained after ammonium sulphate concentration after removal of DTT by gel permeation. 44 and 92% inhibition was observed after incubation with 1 mM *p*-chloromercuribenzoic acid or *N*-ethyl-maleimide respectively (see Experimental). It is therefore likely that TFT has at least one thiol group necessary for its activity which needs to be protected from oxidation. However DTT can also induce a denaturation of TFT in dilute extracts. When the loss of activity in the presence of DTT was monitored using the photometric assay, it was correlated with an increase in 'thioesterase' activity (corresponding to the drift in the baseline before adding tyramine in the cell). DTT was therefore used only in the extraction buffer, mercaptoethanol being used in the following steps.

Partial purification

TFT was purified 46-fold with a 24% yield by DEAE cellulose and hydroxylapatite chromatography (Table 1). The hydroxylapatite column proved especially efficient, the activity being eluted in a single fraction in 0.1 M Pi buffer. The partially purified extract was used to study the specificity of TFT and its affinity for its substrates. The activity of cinnamoyl-CoA derivatives does not significantly differ between the partially purified (Table 2) and the crude extract [1, 2]. Feruloyl-CoA and *p*-coumaroyl-

Table 1. Partial purification of TFT from tobacco leaves

	Total activity (pkat)	Protein (mg)	Specific activity (pkat/mg)	Purification factor
Crude extract	6169	1088	5.6	—
$(NH_4)_2SO_4$	4410	624	7	1.3
DEAE cellulose	2668	188	14.2	2.5
Hydroxylapatite	1491	5.7	260	46.5

Table 2. Relative activity of cinnamoyl-CoA thioesters in the tyramine transferase assay

Substrate	K_m (μM)	Relative activity*
Cinnamoyl-CoA	35.7	100
p-Coumaroyl-CoA	6.2	63
Sinapoyl-CoA	26	54
Feruloyl-CoA	4.9	58
Caffeoyl-CoA	—	0

* V_{max} relative to the V_{max} of cinnamoyl-CoA. TFT activity was measured using the photometric assay in the partialy purified extract (Table 1). Results do not significantly differ from those obtained with the crude extract [1, 2] except for the K_m values of cinnamoyl- and sinapoyl-CoA [1].

Table 3. Relative activity of tyramine analogs in the amine feruloyl tranferase assay

Substrate	Relative activity*
Dopamine	100
Noradrenaline	80
Octopamine	71
Tyramine	67
3-Methoxytyramine	42
Phenylethylamine	11
Homotyramine	7
N-Methyltyramine	0

* V_{max} relative to the V_{max} of dopamine. TFT was purified as described in Table 1.

TFT activity was measured spectrophotometrically in the partially purified extract. A saturating concentration of feruloyl-CoA (25 μM) was used. For structures see Ref. [1].

CoA are the two substrates for which TFT has the highest affinity.

Caffeoyl-CoA is inactive both in the crude [1] and in the purified extract. A large number of tyramine analogues are still active in the feruloyl transferase assay (Table 3) but purification modifies the relative activity of the different amines [1, 2]. Dopamine for example is used more quickly than tyramine after purification. The K_m for tyramine (25 μM) is however still much smaller than the K_m for dopamine (250 μM). This variation of the relative activity of tyramine and dopamine during purification suggested the presence of several isoenzymes in the extract. To test this hypothesis we therefore attempted to separate several active fractions by ion exchange chromatography.

DEAE Trisacryl chromatography

The ammonium sulphate fractionated extract was separated into four active fractions by DEAE Trisacryl

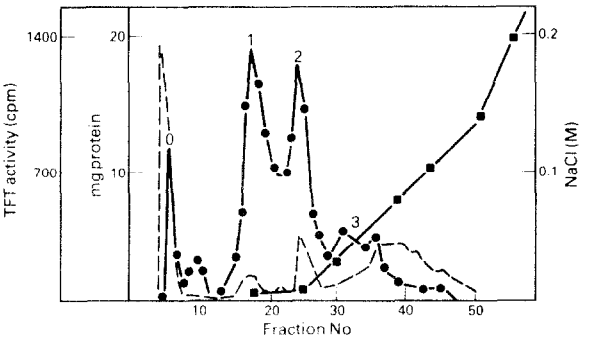

Fig. 1. DEAE Trisacryl chromatography of the ammonium sulphate fractionated extract. Elution was performed at pH 7.7 in a 0.01 M PPi buffer with a NaCl gradient using a flow rate of 0.7 cm/min. 200 ml buffer B were pumped onto the column before starting the gradient. TFT activity was determined using the isotopic assay. Protein was measured using the method of Bradford. NaCl concentration was monitored by titration of Cl^- ions with silver nitrate. (●—●) TFT activity (■---■) protein (■—■) NaCl

chromatography (Fig. 1). The first two fractions (0 and 1 in Fig. 1) correspond to the same protein mixture: they split into two peaks corresponding to fractions 0 and 1 when they are re-analysed on the same column. Fractions 1–3 on the contrary clearly differed in their protein composition, when analysed by polyacrylamide gel electrophoresis. The three fractions still contained a very complex mixture of proteins although fraction 1 and 2 were purified 140- and 58-fold respectively (Table 4).

The pH for optimal activity of the three fractions was compared using the isotopic assay with tyramine and feruloyl-CoA as substrates. Only slight differences were found (7.6, 8.3 and 8 respectively for fractions 1–3). Nevertheless the optima were broad enough to allow measurement of activity in the 3 fractions at pH 8.

When the relative activity of the tyramine analogues in the three fractions was compared (data not shown), no major difference was found, each fraction keeping a broad specificity for phenethylamines (see Table 3). In the three fractions the V_{max} for dopamine is higher than for tyramine but the apparent K_m values for the two amines differ considerably according to the cinnamoy-CoA derivative used as second substrate (Table 5). Fraction 1 and 2 conjugate dopamine much more readily to feruloyl-CoA than to *p*-coumaroyl-CoA. In fraction 3 by contrast,

Table 4. Separation of fractions containing TFT activity by ion exchange chromatography on DEAE Trisacryl

	Total activity (pkat)	Protein (mg)	Specific activity (pkat/mg)	Purification factor
Crude extract	5852	821	7.1	
$(NH_4)_2SO_4$	5653	184	30.7	4.3
DEAE Trisacryl	3850 =			
Fraction 0	627	26	24.1	20
Fraction 1	+ 717	4	181	140
Fraction 2	+ 1378	9.4	147.2	58
Fraction 3	+ 1129	36.8	30.6	15

Table 5. Comparison of the affinity of the enzymes present in the three fractions for tyramine or dopamine using coumaroyl- or feruloyl-CoA as second substrate

Second substrate	K_m values (μM) for tyramine and (dopamine) Fraction 1	Fraction 2	Fraction 3
p-Coumaroyl-CoA	70 (3570)	80 (nd)	45 (131)
Feruloyl-CoA	58.8 (96)	62 (156)	71 (104)

nd:Not determined, even at 10 mM dopamine the activity was hardly detectable using the photometric assay.

Table 6. Comparison of the K_m values (μM) and of the relative activity of CoA thioesters between the three fractions

Substrate	Fraction 1 K_m	R. A	Fraction 2 K_m	R. A.	Fraction 3 K_m	R.A.
Cinnamoyl-CoA	41	100	83	100	10.9	100
Coumaroyl-CoA	4	63	4.2	57	7.6	30
Caffeoyl-CoA	—	0	—	0	—	0
Feruloyl-CoA	7.8	51	26	47	6.8	39
Sinapoyl-CoA	12	63	19.3	85	16.6	50

R. A. = Relative activity (V_{max} relative to the V_{max} of cinnamoyl-CoA).

the nature of the cinnamoyl-CoA moiety does not significantly affect the affinity for the amine. The K_m values and the relative activity of CoA thioesters in the three fractions are shown in Table 6. None uses caffeoyl-CoA as substrate. Two important differences are observed: fraction 2 has a low affinity for feruloyl-CoA by comparison with fractions 1 and 3. On the other hand, fraction 3 has a high affinity for cinnamoyl-CoA.

DISCUSSION

The specific activity of TFT extracted from tobacco leaves is very low even after inoculation with TMV. This makes its purification difficult despite its stability. Among the techniques that we tested to purify TFT, DEAE Trisacryl and hydroxylapatite chromatography proved the more efficient and could be combined. Attempts to use an affinity column of Sepharose 4B on which tyramine had been immobilized by an amide bond were unsuccessful. Most of the proteins including TFT were very strongly retained on the column and could not be eluted (see Experimental), probably because of the very strong hydrophobic interaction between tyramine residues and proteins. The separation of the crude extract into active fractions exhibiting differences of specificity toward the substrates suggests that several enzymes with TFT activity occur in tobacco leaves. The preliminary results presented here suggest that these enzymes still exhibit a wide specificity for phenylethylamines and cinnamoyl-CoA thioesters but differ in their affinity for their substrates. The occurrence of these isoenzymes must however be confirmed by purification. Isoforms of enzymes using cinnamic acids or cinnamoyl-CoA derivatives as substrates have been identified in the phenylpropanoid pathway leading to the synthesis of lignin[7–11]. The difference in the distribution of hydroxycinnamoyl-CoA ligases for example is thought to play a role in the control of the monomeric composition of lignin [12]. It is therefore possible that the identification of TFT isoenzymes would contribute to the understanding of the role of cinnamic acid amides of phenylethylamines inside the phenylpropanoid pathway.

EXPERIMENTAL

Plant material. Nicotiana tabacum cv Xanthi n. c. were grown and inoculated with TMV as described in ref. [1]. 72 hr after inoculation leaves were frozen in liquid N_2 and stored at −80°.

Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of cinnamoyl-*N*-hydroxysuccinimide esters [13].

Detection of TFT activity. TFT was detected and quantified using either the isotopic or the spectrophotometric assay as described in ref. [1].

Extraction and purification of TFT. All work was carried out at 0–4°. TMV inoculated leaves (200 g fr. wt) were blended in 400 ml 0.1 M PPi buffer pH 7.5 containing 5 mM DTT, 1.2% ascorbic acid, 1 mM EDTA and 10 μg/ml α_2 macroglobulin. The homogenate was then centrifuged at 30 000 *g* for 30 min. Protamine sulphate in soln in H_2O (1mg/ml) was then added to the supernatant (0.1 mg/ml protein). After 20 min stirring the extract was centrifuged for 10 min at 30000 *g*. A saturated $(NH_4)_2SO_4$ soln at pH 7.5 was then added to the supernatant to 40% satn, and the mixture stirred for 30 min. After centrifugation at 30 000 *g* for 10 min the ppt. was discarded. Solid $(NH_4)_2SO_4$ was then dissolved in the supernatant to 70% satn and stirred for 30 min. After centrifugation the ppt. was redissolved in the extraction buffer containing 10 mM ME instead of DTT. The extract was then dialysed overnight against 0.01 M PPi buffer pH 8.5 (buffer A) or 7.7 (buffer B) containing 10 mM ME and 1 mM EDTA.

After dialysis against buffer A, the extract was loaded on a DEAE cellulose (Whatman DE 52) column (10 × 2.4 cm) which had been pre-equilibrated in the same buffer. The column was then washed with 60 ml buffer and proteins eluted using a KCl linear gradient in 10 ml fractions with a flow rate of 0.7 cm/min. The active fractions, containing 0.15 to 0.2 M KCl are combined and concd by ultrafiltration using an Amicon P30 membrane. The concd extract (*ca* 10 ml) was then pumped onto a hydroxylapatite (Biorad) column (10 × 2.4 cm) pre-equilibrated in 0.01 M K-Pi buffer pH 7.6 containing 1 mM EDTA and 10 mM ME. A flow rate of 0.7 cm/min was used. Proteins were eluted sequentially using 0.01 M, 0.05 M, 0.1 M and 0.5 M K-Pi buffers. TFT activity was eluted in the 0.1 M fraction, which was then concd by ultrafiltration to *ca* 15 ml. At this stage the activity was stored in the presence of 10% glycerol in liquid N_2.

The extract obtained after $(NH_4)_2SO_4$ fractionation was loaded after dialysis against buffer B on a DEAE Trisacryl M (IBF France) column (15 × 2.4 cm) pre-equilibrated in the same buffer. After pumping 200 ml of buffer B, a linear gradient of NaCl (0–0.2 M) was then applied using a flow rate of 0.7 cm/min to elute the proteins retained on the column (see Fig.1). The different active fractions were then concd by ultrafiltration and stored in 10% glycerol in liquid N_2. Freezing did not alter the properties of TFT shown in Tables 5 and 6.

Affinity chromatography. The tyramine-Sepharose 4B column was prepared according to the manufacturer's instructions, using the *N*-hydroxysuccinimide ester as intermediate. The following treatments were used to attempt to elute TFT from the column:0.5 M KCl, a pH gradient (6 to 8), 3.5 mM tyramine, 20% polyethylene glycol, 20% glycerol, 4 M urea. Among these

treatments only urea eluted part of the proteins retained on the column, but no TFT activity was detected in the eluate.

Effect of sulphhydryl reagents on TFT activity. Aliquots of the enzyme extract were incubated for 1 hr at 20° in the presence of *p*-chloromercuribenzoic acid or *N*-ethylmaleimide (1 mM). After incubation, the mixture was chilled in ice and a 10-fold excess of ME was added to destroy excess reagent [14]. TFT activity was then assayed photometrically using the usual procedure.

Protein was determined using the method of ref. [15]. *Polyacrylamide gel electrophoresis* of proteins was carried out under denaturating conditions at 10% concn.

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