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THE BIOSYNTHESIS OF CINNAMOYLPUTRESCINES IN CALLUS TISSUE CULTURES OF *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco tissue culture; biosynthesis; cinnamic acid amides; putrescine; spermidine; ascorbic acid; enzyme extraction.

Abstract—Extracts of callus of *Nicotiana tabacum* cv Xanthi n.c. catalyse the formation of caffeoylputrescine from caffeoyl-CoA and putrescine. The enzyme caffeoyl-CoA putrescine *N*-caffeoyl transferase is soluble, with a M_r of *ca* 48 000. It is not specific for putrescine since cadaverine, agmatine and spermidine are also conjugated to caffeoyl-CoA in the crude enzymic extract. The coenzyme A derivatives of ferulic, cinnamic, sinapic and coumaric acids also act as substrates in the PCT assay, although they are less active than caffeoyl-CoA. PCT activity is stable *in vitro* when it is extracted with Tris buffer containing 1% ascorbic acid. *In vivo* spermidine is a precursor of *p*-coumaroylspermidine, and also of cinnamoylputrescines. Feeding experiments suggest that putrescine and spermidine can be rapidly interconverted.

INTRODUCTION

Putrescine (1,4-diaminobutane) and the polyamines spermidine and spermine are known to occur in plants both in free and conjugated forms [1, 2]. The main putrescine conjugates which have been identified are *p*-coumaroyl-, caffeoyl- and feruloyl-putrescine, which are also known as cinnamoylputrescines [2, 3]. The relative proportions of free and conjugated polyamines are variable, but it has been reported that up to 90% of the polyamine pool can occur in plants in the conjugated form [4]. Moreover, some plant organs (e.g. flowers or seeds) are known to contain especially high levels of cinnamoylputrescines [1]. In view of the role of polyamines in plant growth and development [5, 6], it is becoming increasingly important to understand the metabolic relationship between bound and free polyamines, and therefore to identify the different enzymes involved in the biosynthesis and the metabolism of the amides.

Since Mizusaki's work on nicotine biosynthesis, it is known that radioactivity from putrescine is rapidly incorporated into cinnamoylputrescines in tobacco callus cultures [7, 8]. This has been confirmed in different tobacco cell lines grown in suspension [3]. Accumulation of cinnamoylputrescines has also been reported to take place in tobacco leaves inoculated with tobacco mosaic virus (TMV) and in the flowers of many plant species [1, 2]. However, despite the diversity of the plant materials which may have been used to study the biosynthesis of these amides, it has proved difficult to detect a putrescine hydroxycinnamoyl–CoA transferase activity *in vitro* [9–11].

Only two enzymes catalysing the formation of cinnamic acid amides in plants have been identified so far. They both use cinnamoyl-CoA derivatives as precursors of the phenolic moiety of the amides. ACT (agmatine *p*-coumaroyl transferase) has been identified in barley seedlings [12, 13]. TFT (tyramine feruloyl transferase) has been found in tobacco leaves [9]. In extracts of *Nicotiana tabacum* cv Xanthi n.c. leaves which had been inoculated with TMV, we occasionaly detected the formation of caffeoylputrescine from [^{14}C]-putrescine and caffeoyl-CoA. This activity was however remarkably labile and we were unable to develop a reliable assay to study its properties *in vitro*. We also occasionaly detected an activity in tobacco callus extracts, without solving the problem of the stability of the enzyme. It was however eventually found that the transferase activity could be stabilized by the addition in the extraction medium of high concentrations of ascorbic acid. This paper describes the characterization and some of the properties of the enzyme caffeoyl-CoA putrescine *N*-caffeoyl transferase (EC 2.3.1-) in crude tobacco callus extracts.

RESULTS

Metabolism of putrescine and spermidine in callus cultures

Mizusaki noticed during tracer studies on nicotine biosynthesis in callus cultures of *Nicotiana tabacum* grown in suspension that radioactivity from [^{14}C]-putrescine was incorporated into at least four ninhydrin positive basic compounds. Three of these have been identified as *p*-coumaroyl-, caffeoyl-, and feruloyl-putrescine [7, 8]. We show that tobacco callus cultures grown on solid medium have comparable metabolism of [1,4-^{14}C]-putrescine and [1,4-^{14}C]-spermidine (Table 1). Radioactivity from putrescine is incorporated into cinnamoylputrescines, with caffeoylputrescine being the most abundantly labelled amide. Radioactivity from putrescine is also incorporated into 4-aminobutyric acid, spermidine and *p*-coumaroylspermidine. This amide was also rapidly labelled when [^{14}C]-spermidine was fed to the callus. These results indicated that callus extracts

should contain a putrescine (and spermidine) hydroxycinnamoyl-CoA transferase and that caffeoyl-CoA was likely to be a good substrate for the enzyme conjugating putrescine. The metabolites labelled from [^{14}C]-spermidine did not differ from those labelled from [^{14}C]-putrescine (Table 1). The interconversion of the two amines could be detected after 2 hr metabolism, before the labelling of the amides.

Biosynthesis of caffeoylputrescine in vitro

First attempts to detect the formation of caffeoylputrescine *in vitro* from caffeoyl-CoA and radioactive putrescine were unsuccessful. Caffeoyl-CoA, like most catechols, is liable to be oxidized in crude enzymic extracts. An attempt was therefore made to protect it from chemical or enzymic oxidation by the addition of various reducing agents, the results are summarized in Table 2. When 28-day-old callus was ground in 0.1 M Tris–HCl buffer containing 1% ascorbic acid at pH 8.5, the supernatant obtained after centrifugation was found to readily catalyse the synthesis of caffeoylputrescine. After centrifugation at 100 000 *g* for 1 hr, all the activity was still in the supernatant. The formation of caffeoylputrescine did not occur without enzyme extract, without one of the two substrates, or in a boiled enzymic extract. Caffeoylputrescine was identified by co-chromatography with the synthetic amide in systems 1 and 2 and by 2D chromato-electrophoresis. The electrophoresis separated the *cis* and *trans* forms. The amide formed *in vitro* was mainly in the *trans* form although trace amounts of the *cis* form was always present. In the absence of enzyme no formation of caffeoylputrescine could be detected after incubation of caffeoyl-CoA with putrescine in the pH range 5 to 11, caffeoyl-CoA being less reactive than acetyl-CoA which reacts spontaneously with putrescine above pH 8 [14]. The formation of radioactive dicaffeoylputrescine was not observed *in vivo* or *in vitro*.

Table 1. Metabolism of [1, 4-^{14}C]-putrescine and [1, 4-^{14}C]-spermidine in tobacco callus cultures

	^{14}C-Putrescine		^{14}C-Spermidine	
Putrescine	70.4%		28.9%	
Spermidine	8.2		47.8	
4-Aminobutyric acid	7.2		2.5	
p-Coumaroylputrescine	1.2		1.0	
Caffeoylputrescine	5.3	10.8	4.6	8.6
Feruloylputrescine	4.3		3.0	
p-Coumaroylspermidine	3.4		12.2	

Results are expressed as % of the total radioactivity absorbed by the callus. After 8 hr feeding *ca* 80% of the supplied radioactivity (37 kBq) was absorbed, with both putrescine and spermidine. After extraction of cinnamic acid amides in MeOH–H_2O–HOAc (50:49:1), the partition of the radioactivity in the different metabolites was studied by 2D chromato-electrophoresis and autoradiography.

Cinnamoylputrescines and 4-aminobutyric acid were identified by co-chromatography with synthetic standards. *p*-Coumaroylspermidine was identified as follow: the fluorescent spot had the characteristic colour of coumaric acid derivatives, which intensified in ammonia, and a mobility in electrophoresis indicating that it was more basic than cinnamoylputrescines but less than spermidine. It was then co-chromatographed with the conjugate formed enzymaticaly between spermidine and *p*-coumaroyl-CoA *in vitro*. Both compounds gave the same double spot after exposure to UV light, due to the cis–trans isomerisation.

Table 2. Effect of reducing agents in the extraction medium on the recovery of PCT activity

Medium used for the extraction of the enzyme PCT activity*	
Tris–HCl	0
Tris–HCl + ascorbate 0.25%	55
Tris–HCl + ascorbate 0.5%	79
Tris–HCl + ascorbate 1%	100
Tris–HCl + ascorbate 2%	100
Tris–HCl + ME 10 mM	54
Tris–HCl + DTT 5 mM	5
Tris–HCl + ascorbate 1% + ME 10 mM	100

PCT activity was estimated in the extraction medium at pH 8.5 (Tris–HCl 0.1 M) using the isotopic assay. After incubation of the enzymic extract, caffeoylputrescine was separated by TLC. When the callus was extracted in the presence of insoluble polyvinylpyrrolidone (1.5 g/g fresh weight) *ca* 30% of the activity found with 1% ascorbate could be detected. Similar results were obtained with activated charcoal (50 mg/g fresh weight). Dehydroascorbic was inactive.

*Expressed as per cent of the activity detected in the presence of 1% ascorbic acid. Activity of PCT in Tris–HCl + ascorbate 1% was 65 pkat per mg protein.

Optimal requirements for putrescine caffeoyl transferase (PCT) activity

The activity detected in the crude extract was stable in the presence of ascorbic acid. Addition of mercaptoethanol (10 mM) was however necessary to retain the activity after concentration with ammonium sulphate. At this stage it was possible to dialyse the enzyme against a buffer which did not contain ascorbate, without losing the enzymic activity. Enzyme activity was maximal at pH 8.5 with 50% activity at pH 7.2 and 10. The apparent K_m for putrescine was 50 μM at 250 μM caffeoyl-CoA in crude extracts. The apparent K_m for caffeoyl-CoA in the same conditions was 10 μM and 1 mM putrescine. No inhibition of PCT activity was observed when the enzyme was assayed in the presence of 1 mM EDTA.

Spectrophotometric assay. The assay used for the determination of agmatine coumaroyl transferase activity [13] was adapted for PCT. The wavelength used to monitor the disappearance of caffeoyl-CoA (400 nm) corresponds to the maximum of the difference spectrum between caffeoyl-CoA and caffeoylputrescine at pH 8.5. It is slightly longer than the λ_{max} of the thioester at pH 8.5 (375 nm). At this pH the phosphohydrolysis of caffeoyl-CoA in a crude enzymic extract can interfere with the spectrophotometric assay, as the cleavage of the phosphodiester bond of coenzyme A leads to a shift in the spectrum or hydroxycinnamoyl-CoA derivatives [9]. This interference however was found to be insignificant in callus extracts. The molar extinction coefficient corresponding to the formation of one mole of caffeoylputrescine is 12 000 M/cm. The spectrophotometric assay was

used during this work to compare the activity of non-radioactive putrescine analogues

Substrate specificity. The relative activity of several di- or polyamines in the caffeoyl transferase assay is shown in Table 3. Putrescine is the best substrate, but the crude enzyme extract can also conjugate cadaverine, agmatine and spermidine. From a physiological point of view it seems unlikely that putrescine and spermidine would be conjugated to cinnamic acids by the same enzyme, since the two amines play distinct biochemical roles. The possible occurrence of several transferases in the extract therefore makes the interpretation of the results shown in Table 3 difficult. Cinnamic acid amides of agmatine or cadaverine have never been identified in tobacco tissues. The fact that these two amines act as substrates may be due to a lack of specificity of the enzymes conjugating putrescine and spermidine.

The relative activity of the different cinnamoyl-CoA derivatives, determined using the isotopic assay, is shown in Table 4. The apparent K_m values for the different thioesters have not been determined because of the complexity of the crude enzymic extract. The concentration of the thioesters (250 μM) in the incubation medium was however probably high enough to give a first indication on the specificity of the transferase. The fact that caffeoyl-CoA is the best substrate confirms the results of the *in vivo* feeding experiments. By contrast the activity of *p*-coumaroyl-CoA *in vitro* does not correspond to the proportion of *p*-coumaroylputrescine formed *in vivo*. It is important to recall however that the activity of *p*-coumaroyl-CoA has been measured using the optimal conditions determined with caffeoyl-CoA as substrate. It is also possible that several isoenzymes with different specificity towards cinnamoyl-CoA derivatives occur in the extract.

Table 3. Relative activity of putrescine analogues in the putrescine caffeoyl-CoA transferase assay

1, 3-Diaminopropane	<1
1,4-Diaminobutane (putrescine)	100
1,5-Diaminopentane (cadaverine)	64
1,6-Diaminohexane	5
1,8-Diaminooctane	<1
Agmatine	53
Spermidine	9
Spermine	<1
4-Aminobutyric acid	<1
Ornithine	<1
Lysine	<1
Arginine	<1

The relative activity of putrescine analogues has been compared spectrophotometrically, using a saturating concentration of caffeoyl-CoA (50 μM). The identity of the products formed in the assay has been checked using radioactive precursors in the case of putrescine, cadaverine, agmatine and spermidine, but not for diaminohexane. After incubation of agmatine both caffeoyl-agmatine and caffeoylputrescine could be detected. This was probably due to the partial hydrolysis of agmatine in the crude extract. The relative activity of agmatine may therefore be overestimated.

<1 means that the activity was below the sensitivity of the assay. 100% corresponds to 83 pkat in a 1 ml cell, i.e. a decrease in A_{400} of 10^{-3}/sec.

Table 4. Estimation of the relative activity of CoA thioesters in the putrescine transferase assay

Caffeoyl-CoA	100%
Feruloyl-CoA	30
Cinnamoyl-CoA	29
Sinapoyl-CoA	10
p-Coumaroyl-CoA	4

The relative activity of the esters was estimated with the isotopic assay using a saturating concentration of putrescine (1 mM) and a high concentration (250 μM) of the esters. Except for caffeoyl-CoA the apparent K_m values of the esters was not determined because of the complexity of the enzymic extract, which probably contains more than one amine transferase. Activity of PCT was 75 pkat per mg protein.

M_r *estimation.* The M_r of PCT was estimated by gel filtration of the crude extract obtained after ammonium sulphate precipitation and dialysis. Using caffeoyl-CoA and putrescine as substrates, maximal activity was detected in fractions corresponding to a M_r of 48 000.

Development of PCT activity in callus cultures

The time course of changes in PCT activity in callus cultures obtained from leaf discs is shown in Fig. 1A. In the conditions used during this experiment the callus grew rapidly without renewing the medium for *ca* 5 weeks (Table 2). Afterwards the cells accumulated phenols which oxidised rapidly. Moreover the cells became hyperhydric, the water content rising to 97% of the fresh weight (Fig. 1B). The PCT activity, expressed on a fresh weight basis, strongly increased during the first two weeks of culture and then declined rapidly. In fact the development of PCT activity almost parallels the changes in protein content in the callus culture which rises for two weeks, before declining to reach very low levels in the hyperhydric cells. It is noteworthy however that the specific activity of PCT is always higher in the callus cultures than in the original leaf discs (see zero time in Fig. 1).

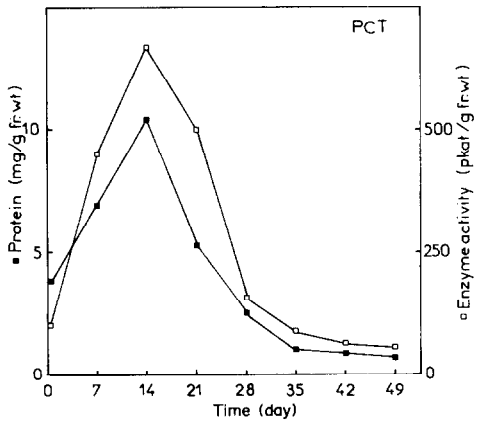

Fig. 1A. Time course of changes in PCT activity, expressed per g of fresh weight, in callus cultures grown from leaf dics on MS medium supplemented with 2, 4-D (2 mg/l) and benzylalanine (0.25 mg/l). PCT activity was measured directly with the isotopic assay in the crude extracts obtained after centrifugation of the tissues homogenates and dialysis.

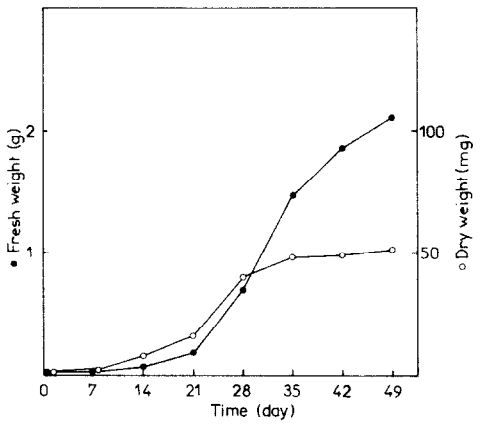

Fig. 1B. Time course of changes in dry and fresh weight of the callus obtained from one leaf disc, grown in the same conditions

In vitro *formation of* p-*coumaroylspermidine*

The fact that radioactivity from [^{14}C]-spermidine was incorporated into a coumaric acid derivative *in vivo* (Table 1) indicated that callus extracts may contain an enzyme conjugating spermidine to *p*-coumaroyl-CoA. This hypothesis was supported by the finding of a low but detectable activity of spermidine in the photometric assay of PCT (Table 3). After incubation of the extract obtained after ammonium sulphate fractionation with [^{14}C]-spermidine and *p*-coumaroyl-CoA at pH 8.5 for 2 hr, a compound, both fluorescent and radioactive, could be detected by TLC in system 2. (Similar compounds were formed after incubation of the enzymic extract with spermidine and caffeoyl- or feruloyl-CoA.) The formation of this compound occurred only in the presence of the two substrates and of the non-denaturated enzyme extract. Analysis by 2D-chromato-electrophoresis of the incubation medium showed that the product was the only radioactive molecule formed. No formation of putrescine from spermidine could be detected *in vitro*. Furthermore the product formed *in vitro* could not be distinguished by 2D-chromato-electrophoresis from the coumaric acid derivative formed *in vivo* (see legend of Table 1).

The exact structure of the spermidine conjugate (assumed to be mono-*p*-coumaroylspermidine from its mobility in electrophoresis) has however not been determined. Spermidine, which is not a symmetrical molecule, can form 3 isomers after conjugation with *p*-coumaric acid (N_1, N_4 or N_8 *p*-coumaroylspermidine). These isomers probably exhibit very close chemical properties and it is not evident that they would be separated even by chromato-electrophoresis. Further work is therefore necessary to determine the structure of the isomer(s) formed both *in vitro* and *in vivo*.

DISCUSSION

The results presented in this paper concerning the *in vivo* incorporation of radioactive putrescine into cinnamoylputrescines in tobacco callus tissue cultures are in agreement with previous work [7, 8]. Although these cultures seemed to be good material to characterize the enzyme conjugating putrescine to cinnamic acids, this has proved difficult, even after the demonstration of the involvment of cinnamoyl-CoA derivatives in cinnamic acid amides synthesis, because of the lability of PCT during the extraction process. The fact that callus extracts can catalyse the formation of putrescine and spermidine conjugates indicates that the crude extract may contain at least two different transferases. The properties of these enzymes, if they both exist, will have to be studied after purification. Even so, the preliminary results obtained with the unpurified extract seem to indicate that the enzyme conjugating putrescine does not differ much from the two other known amine transferases ACT and TFT. The three enzymes are soluble, have a M_r of *ca* 40 to 50.10^3, function at pH values above 6.5, and do not use amino acids as substrates [9, 12, 13]. It is however likely that PCT is not specific for putrescine, when ACT used only agmatine as substrate [13].

One of the most useful consequences of the identification of PCT is that it is now possible to synthesize the radioactive amides of putrescine with a high specific activity. After purification by TLC of samples of caffeoylputrescine obtained enzymatically, yields of up to 80% based on [^{14}C]-putrescine can be obtained. The availability of labelled cinnamoylputrecines should prove useful to study the metabolism of the amides and therefore to understand their metabolic role. The conjugation to cinnamic acids may be a way of regulating the levels of free putrescine in the cell, in which case the amides should, at least in some physiological conditions, undergo hydrolysis and release free putrescine.

Interconversion and turnover of polyamines is well documented in animal cells [15] but has never been studied in plants, although some indications on the conversion of spermidine to putrescine have been published [16, 17]. The results presented in this paper provide some evidence for this interconversion, although they do not give any indication on the mechanism of the reaction. The characterization of PCT is only the first step towards the understanding of the metabolic role of the conjugation of polyamines to cinnamic acids. It is not yet clear whether the amides are only a storage form of polyamines or whether they also exhibit a biological activity, as some experiments done with exogenous cinnamoylputrescines suggest [1]. It is evident however that to answer this question we now need to study not only the metabolism of cinnamoylpolyamines, but also the distribution in the different cell compartments of the bound and free polyamines and of their biosynthetic enzymes.

EXPERIMENTAL

Plant material. Nicotiana tabacum cv Xanthi n.c. plants were grown for *ca* 60 days as described in ref. [9]. Callus cultures were prepared from leaf discs (5 mm in diameter) taken on fully expanded leaves, maintained for 7 weeks on MS medium [18] containing 0.25 mg/l benzyladenine and 2 mg/l 2,4-dichlorophenoxyacetic acid. Leaf discs were grown on 10 ml medium in glass tubes (2.5 cm in diameter) at 25° with a 16 hr photoperiod. Callus was frozen in liquid N_2 and stored at −80°.

Chemicals and radiochemicals. Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters [19], synthesized as described in ref. [20]. Putrescine analogues were commercially available. [1, 4-^{14}C]-putrescine (3.33 GBq/mmol) and [1, 4-^{14}C]-spermidine (3.33 GBq mmol) were obtained from CEA (France). Cinnamoylputrescines were a gift from Dr J. Martin-Tanguy (this institute).

TLC was on cellulose (Merck) using the following solvents: (i) BuOH–EtOH–H_2O (4:1:2) (R_f putrescine 0.05; spermidine 0.02; cinnamoylputrescine 0.57; *p*-coumaroylputrescine 0.42; caffeoylputrescine 0.30; feruloylputrescine 0.34; sinapoylputrescine 0.32; caffeoylcadaverine 0.30; caffeoylagmatine 0.30) (ii) BuOH–HOAc–H_2O (5:2:3) (R_f putrescine 0.25; spermidine 0.10; cinnamoylputrescine 0.77; *p*-coumaroylputrescine 0.69; caffeoylputrescine 0.57; feruloylputrescine 0.64; sinapoylputrescine 0.61; caffeoylcadaverine 0.5; caffeoylagmatine 0.53; *p*-coumaroylspermidine 0.55; caffeoylspermidine 0.51; feruloylspermidine 0.50)

2D-chromato-electrophoresis was performed on cellulose plates (20 × 20 cm). Electrophoresis: HOAc–pyridine–H_2O (5:1:94) for 1 hr at 300 V; mobility relative to a putrescine standard (= 1) trans *p*-coumaroylputrescine 0.40; *trans*-caffeoylputrescine 0.37 (*cis* 0.42); *trans*-feruloylputrescine 0.37; *trans*-*p*-coumaroylspermidine 0.55 (*cis* 0.59); 4-aminobutyric acid 0.51; spermidine 0.9. Chromatography: *n*-BuOH–HOAc–pyridine–H_2O (4:1:1:2) R_f values *p*-coumaroylputrescine 0.72; caffeoylputrescine 0.65; feruloylputrescine 0.59; *p*-coumaroylspermidine 0.58; gamma-aminobutyric acid 0.37; putrescine 0.29; spermidine 0.19.

Feeding experiments. [1, 4-^{14}C]-putrescine and [1, 4-^{14}C]-spermidine were fed *in vivo* to 28 days old callus transferred from the culture tubes to petri dishes. The radioactive solution (37 kBq, 200 μl) was applied at the periphery of each callus with a syringe. After 8 hr, the callus was washed with 5 ml H_2O, then extracted in 3 vol/g fr. wt MeOH–H_2O–HOAc (50:49:1). After centrifugation aliquots (20–50 μl) of the supernatant was analysed directly by 2D-chromato-electrophoresis.

Enzyme preparation. Frozen callus was homogenized at 4° with a pestle and mortar, with sand and 50 mg/g fr. wt. activated charcoal, in 3 vol. 0.1 M Tris–HCl buffer (pH 7.5) containing 10 mM ME, 1 mM EDTA and 1% ascorbic acid w/v). The extract was then centrifuged at 20000 *g* for 15 min and the supernatant dialysed against 0.01 M Tris–HCl buffer pH 8.5 (10 mM ME, 1 mM EDTA). This extract was used to characterize PCT activity. To study the kinetic properties and the specificity of PCT, the activity was concd with solid $(NH_4)_2SO_4$ (65% satn), redissolved in the extraction medium (1 ml/g fr. wt) and dialysed.

Isotopic assay. 10 μl [1, 4-^{14}C]-putrescine (0.74 MBq/ml) was mixed with 25 nmol caffeoyl-CoA (10 μl), 40 μl 0.1 M PPi buffer (pH 8.5), 30 μl enzyme extract and 10 μl 10 mM unlabelled putrescine. The mixture was incubated at 30° for 5 min. The reaction was stopped with 10 μl 17 M HOAc. 10 μl aliquots were analysed by TLC in systems (i) or (ii). Unlabelled caffeoylputrescine, used as a carrier was also spotted on the plate and detected by fluorescence. The isotopic assay was used to determine the optimum pH using PPi, Tris–HCl and $NaHCO_3$–Na_2CO_3 buffers. The same assay was used with spermidine, using 0.1 M Tris–HCl buffer (pH 8.5) instead of PPi buffer.

Spectrophotometric assay. 500 μl enzyme extract was mixed with 500 μl 0.1 M PPi buffer pH 8.5 (10 mM ME) in a 1 ml microcell at 30°. The reference cell contained the same mixture. Caffeoyl-CoA (50 nmol., 20 μl) was added, and the eventual drift in the baseline recorded for 1 min. Afterwards putrescine (1 μmol, 5 μl) was added and the decrease in A_{400} recorded for 1 min.

The M_r of PCT was estimated by gel filtration on an Ultrogel AcA 44 column (50 × 2.6 cm) using a flow rate of 4 cm per hr of 0.01 M PPi buffer containing 1 mM EDTA and 10 mM ME at pH 7.5. The column was calibrated with bovine serum albumin, ovalbumin, myoglobin and cytochrome *c* [21].

Radioactivity was detected on TLC plates after 2D-chromato-electrophoresis by autoradiography. It was then quantified directly on the plate using a Geiger counter. Otherwise radioactivity was detected and quantified on cellulose plates using a TLC radioactivity detector.

Protein was determined according to the method of ref. [22].

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