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PARTIAL PURIFICATION AND PROPERTIES OF *p*-HYDROXYCINNAMOYL-CoA: SHIKIMATE-*p*-HYDROXYCINNAMOYL TRANSFERASE FROM HIGHER PLANTS

BERNHARD ULBRICH and MEINHART H. ZENK

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D4630 Bochum, W. Germany

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Key Word Index—*Cichorium endivia*; Asteraceae; *p*-hydroxycinnamoyl-CoA: shikimate-*p*-hydroxycinnamoyl transferase; 5-*O*-*p*-coumaroyl shikimic acid; cell suspension culture.

Abstract—The partial purification and characterization of *p*-hydroxycinnamoyl-CoA: shikimate-*p*-hydroxycinnamoyl transferase (CST) from cell suspension cultures of *Cichorium endivia* is reported. The enzyme has been purified 126-fold and shows an absolute specificity for *p*-coumaroyl-CoA and shikimic acid. The product of the enzyme reaction has been identified as 5-*O*-*p*-coumaroyl shikimic acid. A wide distribution of CST activity in a variety of plant cell suspension cultures and intact plants was also found.

INTRODUCTION

The occurrence and formation of conjugates between quinic acid and hydroxylated cinnamic acids, such as *p*-coumaric, caffeic and ferulic acids, have been the subject of numerous investigations. However, the occurrence of depsides between shikimic and phenylpropanoid acids has been seldom described [1–3] and only speculations have been made about their biosynthesis [4]. Because of the close chemical relation between both pre-aromatic cyclites the same acylation reaction [5] can be predicted for the formation of both types of conjugates. Thus, one can assume that the phenylpropane moiety has to be activated first, most plausibly in the form of a coenzyme A thiol ester, since the esterification with shikimic acid is an endergonic process. Subsequently, transesterification of the phenylpropane acids to the cyclite takes place under the catalysis of a transferase.

The suggestion that the depsides between quinic and shikimic acid are formed by the action of the same enzyme [4] has been disproven [6]. Studies using an enzyme from *Stevia rebaudiana* clearly showed that there are two separate enzymes, one for the formation of quinate derivatives (hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase = CQT) and the other for shikimate derivatives (hydroxycinnamoyl-CoA; shikimate hydroxycinnamoyl transferase) [6]. In this publication we report the purification and characterization of CST from *Cichorium endivia* and its occurrence in a number of cell suspension cultures and intact plants.

RESULTS

Optical assay for CST

The CST reaction was measured using an optical assay developed for the cleavage (decrease in absorption) of the thiol ester linkage during the transfer reaction [7,8]. Since the CST reaction is reversible, the same extinction coefficient ($\Delta\varepsilon_{342\,nm}$ = 13600cm²/mmol) can be used for the formation of the CoA thiol ester linkage and for the thioclastic split of the ester during *p*-coumaroyl shikimic acid synthesis.

Table 1. Purification of CST

Step	Total volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
1. Centrifuged crude extract	603	511	76.6	0.15	100	1
2. $(NH_4)_2SO_4$, 35–60%	6.9	199	66.8	0.34	87.2	2.2
3. Ultrogel AcA 44	76	38.5	64.8	1.68	84.6	11.2
4. DE52-cellulose	37	12.5	47.3	3.77	61.7	25.1
5. Hydroxylapatite	57.5	4.2	38.8	9.2	50.7	61.3
6. Polyacrylamide gel electrophoresis	0.5	0.4	7.8	18.9	10.4	126.0

The crude extract was prepared from 180 g frozen cell suspension culture of *C. endivia* grown on 4X medium in a culture volume of 20 l.

Purification of CST

Cell suspensions of *C. endivia* were grown for 12 days in a bioreactor, harvested, frozen with liquid nitrogen and stored at −25°. The purification procedure for CST is outlined in Table 1 and resulted in a 126-fold purification of activity. The final fractions were obtained with a 10% yield. The purified CST was free of any thiol esterase and CQT activity.

Characteristics and properties of CST

For the determination of the catalytic properties of the enzyme, the fractions used showed a 61-fold purification after chromatography on hydroxylapatite. As shown in Table 2, the only substrate for CST of *C. endivia* is *p*-coumaroyl-CoA with shikimate as the acyl acceptor molecule. Caffeoyl-CoA and feruloyl-CoA, as well as quinic acid, are inactive. In the presence of coenzyme A and CST, *p*-coumaroyl shikimate is split into *p*-coumaroyl-CoA and shikimic acid. For the reverse reaction, the CST shows an affinity to *p*-coumaroyl shikimate four times less than that to *p*-coumaroyl-CoA in the forward reaction.

Nature of reaction product

The reaction product of the condensation of *p*-coumaroyl-CoA with shikimate was isolated by column chromatography and has been unequivocally determined as 5-*O*-*p*-coumaroyl shikimic acid by ^{1}H NMR. The analysis of the compound is based on the spectra of shikimic acid, the assignment of the ^{1}H NMR signals at δ 5.08, 4.73 and 4.36 to the H-3, H-5 and H-4 protons respectively in the molecule, and the change in chemical shift of the proton when the hydroxyl group, associated with the same carbon atom, is acylated. Thus in the spectra of the compound the signals of H-3 (δ 5.0) and H-4 (δ 4.54) remained at *ca* the same position as in shikimic acid itself, but the signal of H-5 shifted from 4.73 to 6.0. This is clearly consistent with the presence of an acyl group on the hydroxyl group at C-5 of the parent shikimic acid.

The UV spectrum of *p*-coumaroyl shikimic acid shows a sharp absorption maximum at 310 nm and a minimum at 254 nm. From standard plots at 310 nm, the extinction coefficient of *p*-coumaroyl shikimic acid was determined to be $\Delta\varepsilon = 9800\,cm^2/mmol$.

pH Optimum, temperature and MW

The influence of pH on the catalytic properties of CST shows a range of activity between pH 5 and 9. Below pH 5 and above pH 9 there is no detectable activity. The pH optimum is at 6.5 in 0.1 M potassium phosphate buffer. 0.1 M Tris–HCl buffer does not inhibit the enzyme, in contrast to the CQT of *Nicotiana alata* [6], but increases the reaction speed by *ca* 25%.

A maximum initial reaction rate was obtained at 40° and the activation energy was calculated from the Arrhenius plot (Fig. 1). It shows a discontinuity at 19° with apparent energies of activation of 42.2 and 71.1 kJ/mol above and below the break point. At −20° the CST retained 65% of the initial activity in 0.1 M potassium phosphate buffer (pH 6.5) over a period of at least 85 days.

Sephadex G-100 gel filtration and comparison with reference globular-type proteins gave the approximate MW of 58 000 for CST from *C. endivia*. This is in the same order of magnitude as the MW for CQT from both *N. alata* (75 000) and *Stevia rebaudiana* (45 000).

Distribution of CST activity in different plants

Table 3 lists the activity of CST from cell suspension cultures and intact plants. One Gymnospermae family (*Pinus pinea*) and 18 Angiospermae families are represented.

No activity was found in the single Pteridophyta (*Ceratopteris thalictroides*) tested. In the Dicotyledonae, activity was found in 32 species of the 43 cultures tested. The highest levels of CST were found in *Stevia rebaudiana* (Asteraceae), *C. endivia* (Compositae), *Rhazya orientalis* (Apocynaceae) and *Catharanthus roseus* (*Apocynaceae*). The highest specific activities were found in *Catharanthus roseus*, *Nicotiana tabacum* var. *samsun* (Solanaceae) and *Rhazya orientalis*. No activity was detectable in *Cannabis sativa* (Cannabaceae), *Hydrangea macrophylla* (Hydrangeaceae), *Cassia angustifolia* (Papilionaceae), *Trifolium pratense* (Papilionaceae), *Daucus carota* (Apiaceae), *Polygonum tinctorium* (Polygonaceae) or *Morinda citrifolia* (Rubiaceae).

CST activity was also found in a number of intact plants. Young leaves of several species showed relatively lower activities than those obtained from suspension cultures. The highest enzyme levels were found in *Nicotiana tabacum* and *Caranga decorticans* (Scrophulariaceae). No activity was found in *Acer pseudoplatanus* (Aceraceae) and *Syringa josikaea* (Oleaceae).

Table 2. Substrate specificity of CST

Substrate	Constant for	K_m (μM)
Forward reaction		
p-Coumaroyl-CoA	shikimate	39
Shikimate	*p*-coumaroyl-CoA	1200
Reverse reaction		
p-Coumaroyl shikimate	coenzyme A	162
Coenzyme A	*p*-coumaroyl shikimate	119

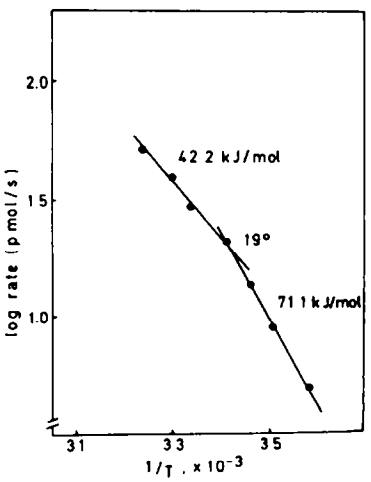

Fig. 1. Arrhenius plot of the variation of CST activity with temperature.

Table 3. CST activity in cell suspension cultures and intact plants

Cell suspension culture	Medium	CST (pkat/mg)	CST (pkat/g fr. wt)
Catharanthus roseus	4X [6]	1516.6	151.6
Nicotiana tabacum var. *samsun*	4X	1513.3	45.0
Rhazya orientalis	4X	1463.3	181.6
Pinus pinea	V M [17]	708.3	6.6
Rheum palmatum var. *tanguticum*	4X	638.3	60.0
Rauwolfia verticilliata	4X	595.0	105.0
Cichorium endivia	4X	523.3	230.0
Nicotiana glauca	4X	521.6	71.6
Datura stramonium var. *tatula*	LS [19]	508.3	53.3
Capsicum sp.	4X	493.3	88.3
Petroselinum sativum	4X	450.0	88.3
Nicotiana glutinosa	4X	378.3	70.0
N. alata	4X	331.6	44.3
Sedum acre var. *americana*	B5 [18]	315.0	25.0
Galium spurium	4X	251.6	90.0
Malus sylvestris var. *domestica*	B5	140.0	86.6
Salix matsudana	4X	113.3	26.6
Forsythia suspensa	4X	103.3	13.3
Geum urbanum	B5	91.6	20.0
Linum usitatissimum	LS	70.0	16.6
Stevia rebaudiana	LS	66.5	207.7
Catalpa bignonioides	LS	58.3	11.6
Phaseolus vulgaris	B5	43.3	3.3
Drosophyllum lusitanicum	4X	40.0	31.6
Coleus blumei	4X	30.0	1.6
Lycopersicon esculentum var. *neglecta*	LS	30.0	3.3
Rubia tinctorum	4X	30.0	10.0
Juglans regia	B5	26.6	28.3
Coffea arabica	4X	25.0	3.3
Solanum tuberosum	4X	10.0	3.3
Pimpinella anisum	4X	8.3	10.0
Agrostemma githago	4X	5.0	1.6
Intact plants			
Nicotiana tabacum		41.6	91.6
Lonicera glaucescens		18.3	43.3
Caragana decorticans		15.0	80.0
Salix purpurea		11.6	35.0
Betula maximowiziana		10.0	30.0
Padus racemosa		6.6	26.6
Lycopersicon esculentum		5.0	5.0
Betula tianschanica		3.3	6.6

DISCUSSION

During the present study CST activity was widely encountered in protein extracts from cell cultures as well as from intact plants. This suggests that the product of the reaction catalysed by CST, namely 5-*O*-*p*-coumaroyl shikimate, occurs widely. Up to now, however, this compound has not been discovered in the plant kingdom. The discovery of an enzyme for a given reaction prior to the occurrence of its reaction product is unusual. This could mean either that little attention has been paid to the shikimate depsides, or more likely, that *p*-coumaroyl-shikimate may act as an acyl donor for an, as yet, unknown aceylation reaction involved in phenylpropane metabolism. Shikimate derivatives of substituted cinnamic acids have only been found in the case of dactylifric acid which occurs in different Palmae species [3,9]. However, this caffeic acid derivative cannot be a direct reaction product of CST since this enzyme is specific for *p*-coumaroyl-CoA and does not act on caffeoyl-CoA (Fig. 2).

Since only caffeoyl [3,9] and feruloyl [10] shikimate derivatives have been found to occur in nature so far, the question remains open whether these products are catalysed by specific transferases using the substrates caffeoyl- or feruloyl-CoA or whether the less likely possibility [6], successive substitution of the aromatic ring of the preformed *p*-coumaroyl-shikimate, occurs. To answer this question it will be of interest to screen tissue extracts for transferase activity involving shikimate as an acceptor.

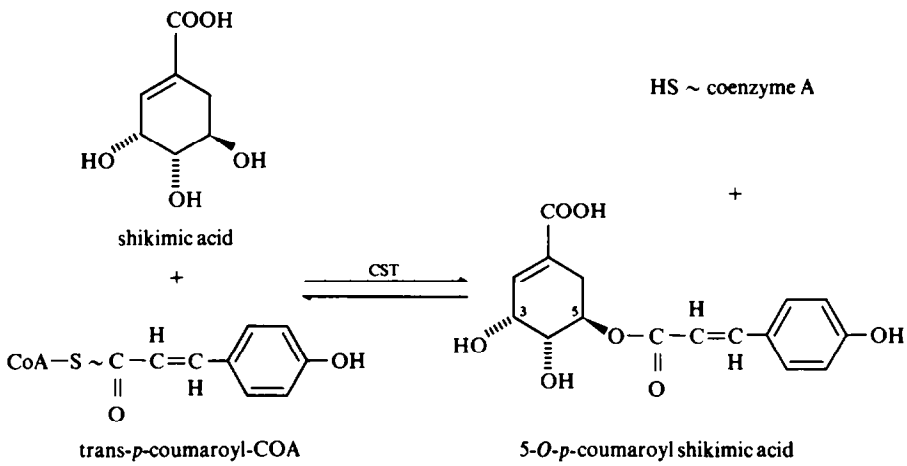

Fig. 2. Reaction scheme of CST from *Cichorium endivia*.

EXPERIMENTAL

Plant sources and biochemicals. Plant cell cultures were provided by our cell culture laboratory. Cells of *C. endivia* were grown axenically in a 20 l. bioreactor using 4X-medium + 2% sucrose [6] over a period of 12 days at room temp. with continuous aeration (200 l./hr). The tissue was harvested, frozen with liquid N_2 and stored at −25°. The intact plants were provided by the University botanical garden. Biochemicals were obtained from Boehringer, Mannheim; hydroxycinnamoyl-CoA thiol esters were synthesized using enzymatic methods [11]. Previously published extinction coefficients were used [6]. Materials for chromatography were obtained from Pharmacia (Sephadex G and LH), LKB (Ultrogel), Whatman (DE 52-cellulose), BIO RAD (hydroxylapatite and Dowex 50W X8), Macherey-Nagel (Polyamide SC 6), Hedinger (Polyclar AT) and from Merck (Avicel for column chromatography).

Enzyme prepn. Frozen cell suspension (180 g) was homogenized for 60 sec with the same wet wt of Polyclar AT and 600 ml of buffer (0.1 M Tris–HCl + 0.25 M sucrose + 0.001 M EDTA + 0.02 M 2-mercaptoethanol; pH 8) [4]. The brei was filtered through 4 layers of cheesecloth, clarified by centrifugation at 27 000 *g* for 30 min and the supernatant made up to 35% satn with $(NH_4)_2SO_4$. The resultant protein ppt. was centrifuged at 27 000 *g* for 30 min. The supernatant was then made up to 60% satn with $(NH_4)_2SO_4$, centrifuged at 27 000 *g* for 20 min and the resulting pellet resuspended in 7 ml 0.1 M Tris–HCl (pH 8.2) and desalted on an Ultrogel AcA 44 column (92 × 2.5 cm; linear flow rate: 6.1 cm/hr) using the same buffer for elution. The CST fractions were pooled and applied to a DEAE-cellulose column (DE 52 18 cm × 1.5 cm; pump flow rate: 30 ml/hr). The enzyme was eluted with 500 ml 0–0.2 M KCl gradient in 0.1 M Tris–HCl (pH 8.2). The partly purified enzyme fractions were pooled, dialysed against 20 l. 0.005 M K-Pi buffer (pH 7.1) and applied to an hydroxylapatite column (4 × 0.9 cm; pump flow rate: 14 ml/hr). CST was eluted with 200 ml 0.005–0.1 M K-Pi buffer (pH 7.1). The purified fractions were pooled and characterized. One quarter of this was further purified by polyacrylamide gel electrophoresis (PAGE). PAGE was carried out using Tris–glycine (pH 8.3; 3 g Tris + 14.4 g glycine 1000 ml H_2O) as the buffer system with a 6% acrylamide gel [12]. Conc enzyme solns (0.25 ml) were applied to gels (10 × 0.6 cm) and PAGE was performed at 3 mA/gel. The gels were sliced into 2.5 mm sections for optical assay of CST and protein (Fig. 3).

Optical assay. Forward reaction 50 μmol K-Pi buffer (pH 6.5): 10 nmol hydroxycinnamoyl-CoA thiol ester; 2 μmol shikimic acid and 50 μl diluted enzyme in a final vol. of 0.5 ml were incubated at 30°. The decrease in *A* of the reaction mixture was followed at 340 nm. Reverse reaction: 50 μmol K-Pi buffer (pH 6.5); 0.2 μmol CoA; 0.1 μmol *p*-coumaroyl shikimic acid and 50 μl diluted enzyme in a total vol. of 0.5 ml were incubated at 30°. The increase in *A* was measured at 340 nm. In both assays the change in *A* was linear over a period of at least 10 min. Protein was determined according to the modified standard Lowry procedure [13–15] and in high purified fractions at 260/280 nm [16]. In both cases BSA was used as standard.

Nature of reaction product. Enzymatic synthesis of *p*-coumaroyl shikimic acid was performed in a batch using hydroxycinnamate: coenzyme A ligase from *Pseudomonas putida* [11] and CST from *C. endivia*: 8 nmol K-Pi buffer (pH 7); 150 μmol $MgCl_2$; 150 μmol ATP (pH 7); 100 μmol CoA (pH 7); 100 μmol *trans-p*-coumaric acid (pH 7); 150 μmol shikimic acid (pH 7); 200 nkat ligase, 146 nkat CST and H_2O were added (80 ml total vol.). The mixture was incubated at 35° for 6 hr in the dark. CST was added to the mixture after the 1st hr of reaction time. The *p*-coumaroyl shikimic acid was extracted into EtoAc after acidification and the extract applied to a Sephadex LH-20 column (85 × 1.5 cm; linear flow rate: 17 cm/hr; elution with H_2O). *p*-Coumaroyl shikimic acid-containing fractions were

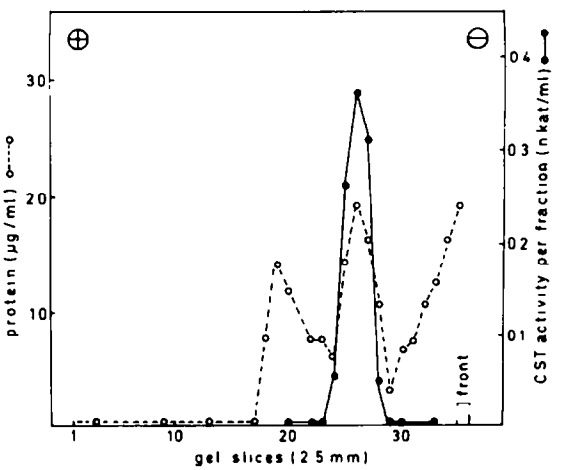

Fig. 3. Separation of CST activity on discontinuous polyacrylamide gel electrophoresis enzyme activity (●——●) and protein (○- - -○) in 2.5 mm slices of the 6% gel.

pooled and lyophilized. The dry residue was dissolved in H_2O and applied to a Dowex 50 W × 8 column (5.5 × 0.9 cm) and eluted with H_2O. The eluate was lyophilized a second time and the white powder dissolved in MeOH and chromatographed on Avicel plates (20 × 20 cm; 1 mm thickness) with $CHCl_3$ shaken with 0.2 vol. of H_2O–MeOH–HCO_2H (21:125:4) as solvent. The *p*-coumaroyl shikimic acid band, easily detected by its blue UV fluorescence at 354 nm, was eluted with MeOH. The yield was 22 mg (68% with regard to *p*-coumaric acid). ^1H NMR studies (220 MHz) were done on the isolated *p*-coumaroyl shikimic acid dissolved in Py-d_5.

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