Phytochemistry, 1979, Vol. 18, pp. 929–933. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0601-0929 $02.00/0

PARTIAL PURIFICATION AND PROPERTIES OF HYDROXYCINNAMOYL-CoA: QUINATE HYDROXYCINNAMOYL TRANSFERASE FROM HIGHER PLANTS

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(*Received* 5 *October* 1978)

Key Word Index—*Nicotiana alata*; Solanaceae; *Stevia rebaudiana*; Asteraceae; chlorogenic acid biosynthesis; cell cultures.

Abstract—The partial purification and characterization of hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (CQT) from two plant sources growing as cell cultures are reported. The enzymes have been purified 50- and 16-fold, respectively, and show an absolute specificity for *p*-coumaroyl-CoA and caffeoyl-CoA as well as for quinate, and are responsible for the synthesis of *p*-coumaroylquinate and caffeoylquinate (chlorogenic acid). The distribution of this transferase activity in a variety of plant cell cultures and differentiated plants is reported.

INTRODUCTION

The biosynthesis of chlorogenic acid [3-*O*-caffeoyl-D-quinic acid] (old numbering used throughout), one of the most widespread phenolic compounds in the plant kingdom, has been a controversial issue in the past [1]. Several pathways have been proposed on the basis of *in vivo* precursor feeding experiments. Chlorogenic acid was reported to be formed by esterification of quinate with cinnamic acid [2] followed by successive hydroxylations. On the other hand, both caffeic [4, 5] and *p*-coumaric acid have also been found to serve as direct precursors in chlorogenic acid formation in intact plants. In addition, unidentified carbohydrate esters of cinnamic acids had been postulated as intermediates [3] in the esterification reaction.

Based on the fact that the esterification of quinic acid with a hydroxycinnamoyl moiety is an endergonic process, the phenylpropane unit most likely requires activation of the carboxyl group. Using an enzyme preparation of *N. alata* cell suspension cultures [6], it was possible to demonstrate unequivocally that chlorogenic acid biosynthesis involves the enzymatic transfer of the caffeoyl moiety of caffeoyl-CoA to quinate. Subsequently, a partially purified enzyme from ripening tomatoes was used to further characterize the enzymological aspects of this reaction [7]. This enzyme, which was named hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase [7] (CQT [8]) was shown to be light-induced [8, 9] in buckwheat, whereas in potato tuber discs [10] it was found that cinnamic acid levels mediate the light-stimulated increase in CQT. Thus CQT represents an interesting enzyme in developmental plant physiology.

We now report on the partial purification and properties of this enzyme from two plant sources as well as its distribution in a variety of differentiated plants and cell suspension cultures.

RESULTS

Optical assay for CQT

The CQT reaction was followed using an optical assay developed for the cleavage (decrease in absorption) of the thiol ester linkage during transfer reactions [12, 13]. However, since the reaction product catalysed by CQT, namely *p*-coumaroyl quinate or chlorogenate, absorbs in the long UV region as do the corresponding CoA esters, the absorption coefficient of this transfer reaction had to be determined under these conditions. It was found that the $\Delta\varepsilon_{342\,nm}$ between *p*-coumaroyl-CoA and *p*-coumaroyl quinate was 13600 cm^2/mmol and between caffeoyl-CoA and chlorogenate 13000 cm^2/mmol at 360 nm. Both coefficients were determined at pH 7.0. It had previously been shown [7] that the CQT reaction was reversible; thus the same extinction coefficient can be used for the formation of the CoA thiol ester linkage or for the thioclastic split of the ester during *p*-coumaroyl quinate or chlorogenate synthesis. *p*-Coumaroyl-CoA was chosen as substrate for the forward reaction because of its greater stability.

Purification of CQT

Suspension cells of *Nicotiana alata* and *Stevia rebaudiana* were grown for *ca* 17 days, harvested, frozen with liquid nitrogen and stored at −20°. The purification procedures for CQT are outlined in Tables 1 and 2 and resulted in a 50- and 16-fold purification of this activity, and the final fractions were obtained in 3.3 and 7.4% yields, respectively. The elution profile of CQT from *S. rebaudiana* is shown in Fig. 1. CQT from *Stevia* was eluted from the DEAE column at 0.055 M KCl while that of *Nicotiana* was eluted by 0.09 M KCl. Both values are in agreement with the values obtained for the enzyme from tomato (0.065 M) [7]. The partially purified CQT enzymes from both sources reported here are, however,

Table 1. Purification of CQT

Step	Total volume (ml)	Total protein (ml)	Total activity (nkat)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
1. Centrifuged crude extract	275	1200	69.2	0.058	100	1
2. Dowex 1	260	1124	86.1	0.077	124	1.3
3. $(NH_4)_2SO_4$	5.5	213	36.6	0.172	53	3
4. Sephadex G 200	5.0	0.76	2.3	3.0	3.3	51

Crude extract was prepared from 100 g frozen cell suspension culture of *Nicotiana alata* grown on LS in a culture volume of 2 l.

completely free of any thiolesterase activity. It can also be seen that the enzyme activity which transfers the *p*-coumaroyl moiety to quinate (CQT) is clearly separable from a second enzyme activity which uses shikimate as the sole acceptor for the phenylpropane unit. This observation led to the discovery of a second enzyme *p*-coumaroyl-CoA: shikimate *p*-coumaroyl transferase (CST), which will be reported separately [11].

Characteristics and properties of CQT

For the determination of the catalytic properties of the enzyme, those fractions showing maximal purification were used.

As shown in Table 3, the only substrates for this enzyme were *p*-coumaroyl-CoA, caffeoyl-CoA and quinate as the acceptor. From the results obtained here, it is likely that the tomato preparation contained a second enzyme (CST) in addition to the CQT. Surprisingly, however, feruloyl-CoA and shikimate are also completely inactive in this transfer reaction, since activity with both substrates has been reported from a CQT preparation of *Lycopersicon* [7]. Myo-inositol and glucose are inactive as acyl acceptors in this reaction.

Table 2. Purification of CQT

Step	Total volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Recovery (%)	Purification (-fold)
1. Centrifuged crude extract	160	792	356	0.45	100	—
2. $(NH_4)_2SO_4$ fractionation	5.8	278	284	1.02	79.7	2.3
3. Sephadex G 200 chromatography	50	36	101	2.81	28.4	6.3
4. DEAE-cellulose chromatography	15	3.5	26.2	7.48	7.4	16.6

Crude extract was prepared from 53 g frozen cell suspension culture of *Stevia rebaudiana* grown on LS in a culture volume of 2 l.

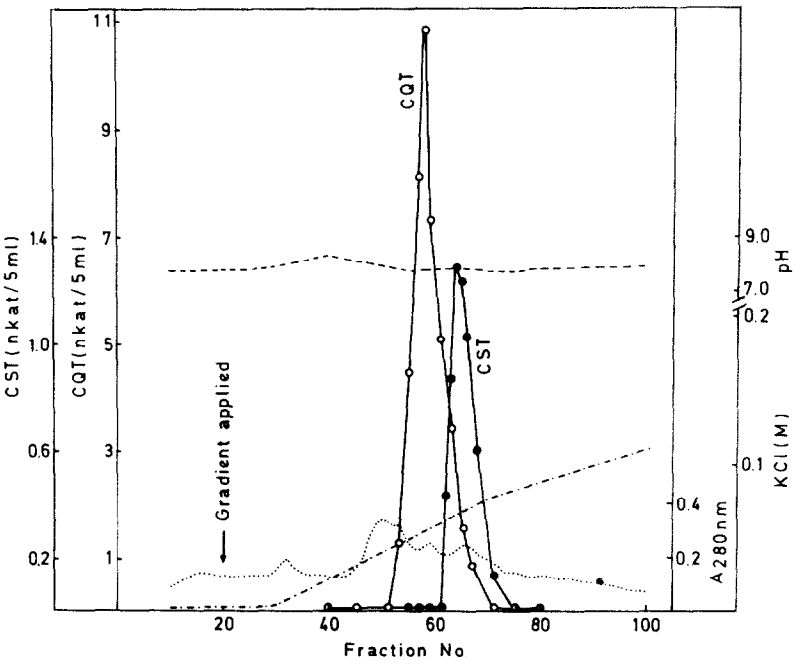

Fig. 1. Localization of CQT and CST from *Stevia rebaudiana* cell suspension culture on a KCl gradient on DEAE-cellulose. ○—○, CQT (pkat *p*-coumaroyl-CoA transferred to quinic acid/5 ml fraction) peak at 55 mM KCl; ●—●, CST (pkat *p*-coumaroyl-CoA transferred to shikimic acid/5 ml fraction) peak at 68 mM KCl; ———, pH; – – –, gradient KCl (M); . . . ., rel. protein $A_{280\,nm}$

Table 3. Substrate specificity of CQT

Source of enzyme		*S. rebaudiana*	*N. alata*
Substrate	Constant for	K_m (μM)	K_m (μM)
Forward reaction			
p-Coumaroyl-CoA	quinate	80	6
Caffeoyl-CoA	quinate	43	29
Feruloyl-CoA	quinate	NR	NR
Quinate	*p*-coumaroyl-CoA	2170	392
Quinate	caffeoyl-CoA	890	700
Quinate	feruloyl-CoA	NR	NR
Shikimate	*p*-coumaroyl-CoA	NR	NR
Shikimate	caffeoyl-CoA	NR	NR
Shikimate	feruloyl-CoA	NR	NR
Reverse reaction			
Coenzyme A	*p*-coumaroyl quinate	294	—
Coenzyme A	chlorogenate	—	57
p-Coumaroyl quinate	coenzyme A	168	—
Chlorogenate	coenzyme A	620	61
Dephospho-CoA	*p*-coumaroyl quinate	5000	—
Pantetheine	*p*-coumaroyl quinate	NR	—
Cysteamine	*p*-coumaroyl quinate	NR	—

NR = No reaction.

From the apparent K_m value, the *N. alata* enzyme shows a greater affinity for *p*-coumaroyl-CoA than for caffeoyl-CoA, whereas the *S. rebaudiana* enzyme shows the highest affinity for caffeoyl-CoA.

In the presence of CoA and CQT, chlorogenate and *p*-coumaroyl quinate are split to the corresponding CoA thiolesters. For the reverse reaction, the CQT from *Stevia* shows a greater affinity for *p*-coumaroyl quinate than for chlorogenate. Dephospho-CoA can serve as an acceptor (6% of CoA) but pantetheine, *N*-succinyl cysteamine or glutathione are inactive.

Identification of the reaction product

The reaction product of the condensation of caffeoyl-CoA with quinate has uniequivocally been determined as being chlorogenate (3-*O*-caffeoyl quinate) by a previously described procedure [6].

pH Optimum, temperature and MW

The enzyme mediated catalysis in the forward reaction using *p*-coumaroyl and caffeoyl-CoA as substrate shows a range of activity between pH 5.0 and 8.5. pH Optimum is at pH 7.0 in 0.1 M potassium phosphate buffer. 0.1 M Tris–HCl buffer, pH 7.0, inhibits the *N. alata* enzyme by 65% but the *S. rebaudiana* enzyme is unaffected.

A maximum initial reaction rate was obtained at 52° for the *Nicotiana* and at 42° for the *Stevia* enzyme. Activation energy was calculated at 13.8 and 12.2 kcal/mol, respectively. At −18° both enzymes are stable over a period of at least 30 days.

Sephadex G 100 chromatography and comparison with reference proteins showed the MW of the *N. alata* enzyme to be 75000 and of the *S. rebaudiana* enzyme to be 45000.

Distribution of CQT within different plants

Table 4 shows the CQT activity from cell suspension cultures representing 17 Angiospermae families. No activity was found in the single Pteridophyta (*Ceratopteris thalictroides*) and Gymnospermae (*Pinus pinea*). In the Dicotyledonae, however, activity was found in 31 species of the 41 cultures tested. The highest levels of CQT were found in *S. rebaudiana* and *Malus sylvestris*. Unfortunately we were not able to establish actively growing cell suspension of *Malus*; therefore the second CQT was isolated from *N. alata*, the third most active culture. CQT activity was also found in a number of differentiated plants. Young leaves of several species showed activities comparable to those obtained from suspension cultures. Two of the highest enzyme levels were found in leaves of *Lycopersicon esculentum* cv Moneymaker (200 pkat/g fr. wt) and *K. tabacum* (155 pkat/g fr. wt).

Table 4.

Differentiated plants		CQT pkat/g fr. wt	pkat/mg
Lycopersicon esculentum		201.6	196.6
Nicotiana tabacum		155.0	71.6
Betula maximowiziana		73.3	26.6
Betula tianschanica		18.3	8.3
Lonicera glaucescens		16.6	6.6
Cell suspension cultures	**Medium**	**CQT pkat/g fr. wt**	**pkat/mg**
Nicotiana alata	4X	35.0	241.6
Rauwolfia verticilliata	4X	31.6	176.6
Rhazya orientalis	4X	18.3	156.6
Stevia rebaudiana	LS*	223.3	135.0
Catharanthus roseus	4X	13.3	131.6
Nicotiana glacua	4X	16.6	116.6
Malus sylvestris var. *domestica*	B5	55.0	90.0
Nicotiana tabacum cv samsun	4X	1.6	70.0
Coffea arabica	4X	8.3	65.0
Trifolium pratense	4X	8.3	55.0
Rheum palmatum var. *tanguticum*	4X	5.0	53.3
Cichorium endivia	4X	21.6	50.0
Daucus carota	B5	8.3	30.0
Linum usitatissimum	LS	6.6	28.3
Cassia angustifolia B5 + coconut milk		1.6	26.6
Lycopersicon esculentum var. *neglecta*	LS	3.3	23.3
Coleus blumei	4X	1.6	23.3
Petroselinum sativum	4X	5.0	21.6
Galium spurium	4X	5.0	15.0
Nicotiana glutinosa	4X	1.6	11.6
Catalpa bignonioides	LS	1.6	10.0
Capsicum sp.	4X	1.6	8.3
Salix matsudana	4X	1.6	6.6
Agrostemma githago	4X	3.3	6.6
Drosophyllum lusitanicum	4X	5.0	6.6
Cannabis sativa	B5	5.0	5.0
Solanum tuberosum	4X	1.6	5.0
Juglans regia	B5	3.3	3.3

*LS = Linsmaier Skoog medium.

DISCUSSION

The properties of the enzymes described herein reveal them to be the final catalysts in the formation

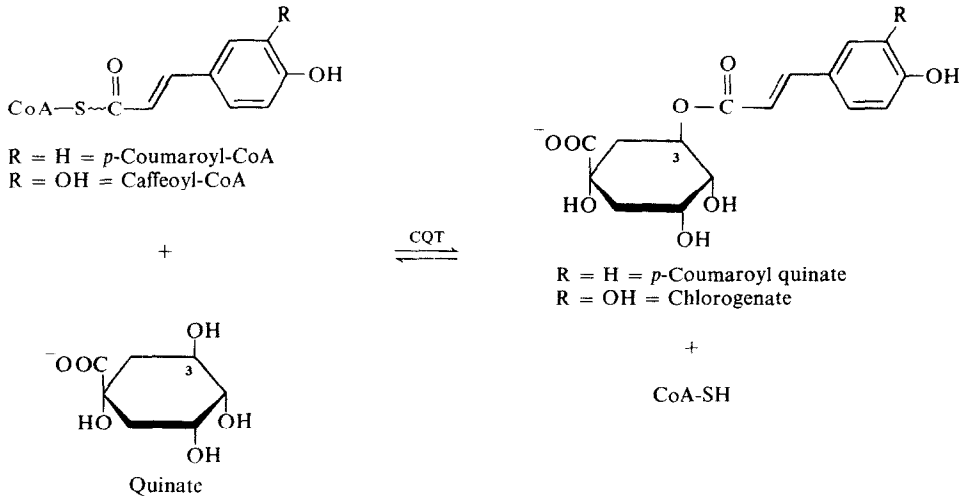

Fig. 2. Reactions catalysed by CQT.

of chlorogenic and 3-*p*-coumaroylquinic acid in higher plants (Fig. 2). The wide distribution of this enzyme in tissue cultures and differentiated plants correlates well with the practically ubiquitous occurrence of these phenolic compounds in higher plants. The question so often posed [7], which of the two pathways to chlorogenic acid is the 'more likely', either acylation of quinate by caffeoyl-CoA or the formation of *p*-coumaroyl quinate and subsequent oxidation of the coumaroyl moiety, is futile [14]. Both pathways seem to exist, as it now also turns out to be the case in flavonoid biosynthesis [15]. The fact that the *Stevia* enzyme shows a higher apparent K_m value for caffeoyl-CoA than for *p*-coumaroyl-CoA supports this view. In CQT from the Solanaceae the reverse appears to be true. Research on chlorogenic acid biosynthesis of carrot suspension cultures [16] also supports our view that caffeoyl rather than *p*-coumaroyl-CoA can act as acyl donor for this depside. It can therefore be assumed that the actual pool sizes of either cinnamoyl-CoA substrate at a given time at the site of the location of CQT will determine which substrate is used for chlorogenic acid synthesis.

The partly purified CQT enzymes reported here do not catalyse the condensation of feruloyl-CoA with quinate. However, since this compound is known to occur in plants [17, 18] it is also likely that other quinate transferases with different substrate specificities exist. A single case has been reported in tomatoes, for instance, where CQT activity was demonstrated using feruloyl-CoA as a substrate [7]. It is, however, not known whether this is a different enzyme or whether the CQT in tomato possesses a different substrate specificity.

The CQT enzymes investigated here have an absolute specificity for quinate; shikimate is totally inactive as is glucose and also myo-inositol, of which a *p*-coumaroyl ester has recently been found [19].

An important question remains; by which mechanism are the isomeric monosubstituted quinic acids, e.g. 4-*p*-coumaroyl quinate [20] and the disubstituted quinate, e.g. 3,5-dicaffeoylquinate [21] formed? The 4-*O*-isomers are most likely synthesized by an enzymatic transesterification process originating from the 3-*O*-isomers [22]. In the case of 3,5-dicaffeoylquinic acid there is evidence for a caffeate substitution of chlorogenic acid in the 5-position [23]. The reversible nature of the CQT reaction reported by Rhodes and Wooltorton [7] and in this paper may partly explain the observed metabolic turnover of chlorogenate [24] in plant tissue as well as 'de-esterification' reactions frequently encountered [5, 23].

EXPERIMENTAL

Plant cell cultures were provided by our cell culture laboratory. The suspension cells were grown at 24°, under 500 lx continuous incandescent light and were shaken at 100 rpm. B 5 [25] was used as standard medium in most cases supplemented with 0.5; 2.0; 0.5; 0.2 mg/l. IAA, 2,4-D, NAA and kinetin (4 × medium). Tissue was grown for 2–3 weeks, harvested, frozen with liquid N_2 and stored at −20°.

Differentiated plants were provided by the University botanical garden. Biochemicals were from Boehringer, Mannheim; hydroxycinnamoyl-CoA thiolesters were synthesized using chemical [13] or enzymatic [26] methods. Previously published extinction coefficients were used.

Enzyme preparation. 50–100 g of frozen suspension cells were homogenized with the same weight of Polyclar AT and 2 vols. of buffer (0.1 M Tris–HCl pH 8.0 containing 0.25 M sucrose, 1 mM EDTA-Na_2 and 20 mM 2-mercaptoethanol) [7] for 60 sec. The brei was filtered through 4 layers of cheesecloth and centrifuged for 30 min at 40000 *g*. The crude extract was stirred for 20 min with 1/10 (w/w) Dowex 1 × 8 (borate form) and then filtered. A 40–80% ammonium sulfate cut was made and the resulting pellet was dissolved in 0.005 M Tris–HCl buffer, pH 7 and applied to a Sephadex G 200 column (superfine; 68 × 2.5 cm). The desalted transferase fractions were pooled and applied to a DE52-cellulose column (Whatman) and the enzyme eluted with 500 ml of a 0–0.2 M KCl gradient (in 0.005 M Tris–HCl, pH 7.5).

Optical assay. (i) Forward reaction: 50 μmol KPO_4^{-2}, pH 6.5 or 7.0, 10 nmol hydroxycinnamoyl-CoA ester, 2 μmol quinate and enzyme (0.75–2.4 μg protein) in a final vol. of 0.5 ml were incubated at 35° and *A* at the appropriate wavelength was followed. Under these conditions a decrease of $E = 0.01$ at 342 nm corresponds to the formation of 0.735 nmol *p*-coumaroyl quinate. (ii) Reverse reaction: 0.1 μmol chlorogenate was incubated in the presence of 50 μmol KPO_4^{-2} buffer pH 7.0, 0.2 μmol CoA and enzyme (*ca* 20 μg protein) in a total vol. of 0.5 ml at 35°. An increase of $E = 0.01$ at 360 nm corresponds to the formation of 0.769 nmol caffeoyl-CoA. In both

reactions the change in absorbance was linear over a period of at least 5 min.

Protein was determined according to standard Lowry procedure [27] but in purified fractions at 260/280 nm. In both cases bovine serum albumin was used as standard.

Nature of the reaction product. Rigorous proof for the identity of the reaction product as 3-*O*-chlorogenic acid has been previously given [6]. For the determination of substitution in the mono-*p*-coumaroyl quinate series, the reaction product was co-chromatographed with isomers of known configuration [28]. Caffeoyl-CoA formed in the reverse reaction was identified as previously published [13].

Acknowledgements—We cordially thank Dr. E. Haslam, Sheffield, for a gift of authentic 3, 4 and 5 *p*-coumaroyl quinic acid. We thank Prof. Mansell, Tampa, for his kind help in the preparation of this manuscript. This research was supported by the Deutsche Forschungsgemeinschaft.

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