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THE ENZYMIC CONVERSION OF HYDROXYCINNAMIC ACIDS TO *p*-COUMARYLQUINIC AND CHLOROGENIC ACIDS IN TOMATO FRUITS

MICHAEL J. C. RHODES and LEONARD S. C. WOOLTORTON
A.R.C. Food Research Institute, Colney Lane, Norwich NR4 7UA, England

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Abstract—Two enzymes thought to be involved in the biosynthesis of chlorogenic acid have been separated and purified by ion exchange chromatography and their properties studied. These two enzymes, *p*-coumarate CoA ligase and hydroxycinnamyl CoA:quinate hydroxycinnamyl transferase, acting together catalyse the conversion of *p*-coumaric acid to 5′-*p*-coumarylquinic acid and of caffeic acid to chlorogenic acid. The ligase has a higher affinity for *p*-coumaric than for caffeic acid and will in addition activate a number of other cinnamic acids such as ferulic, isoferulic and *m*-coumaric acids but not cinnamic acid. The transferase shows higher activity and affinity with *p*-coumaryl CoA than caffeyl CoA. It also acts with feruloyl CoA but only very slowly. The enzyme shows high specificity for quinic acid; shikimic acid is esterified at only 2% of the rate with quinic acid and glucose is not a substrate. The transferase activity is reversible and both chlorogenic acid and 5′-*p*-coumarylquinic acids are cleaved in the presence of CoA to form quinic acid and the corresponding hydroxycinnamyl CoA thioester.

INTRODUCTION

Chlorogenic acid (5′-*O*-caffeyl-D-quinic acid*) is one of the most widely distributed polyphenols in higher plants [1–3]. Three pathways of biosynthesis have been proposed. Levy and Zucker [4] proposed that in the potato, esterification occurs at the cinnamic acid level followed by successive hydroxylations to form chlorogenic acid. Kojima *et al.* [5,6] presented evidence that the glucose ester of cinnamic acid was an intermediate between cinnamic acid and cinnamylquinate in the pathway. Kojima *et al.* [5] proposed, however, an additional route in which quinate ester formation occurs at the *p*-coumarate level. Steck [7] using tobacco leaf disks proposed two separate pathways, one in which esterification occurs at the level of *p*-coumaric acid while in the other at the level of caffeic acid, but his data suggested that the route via *p*-coumarylquinic acid was quantitatively more important. In all these proposals, enzymic evidence for at least one of the steps was lacking.

Recently, Stöckigt and Zenk [8] have shown that a crude enzyme preparation from tobacco cell suspension cultures catalysed the synthesis of chlorogenic acid from caffeyl CoA and quinic acid, thus indicating the involvement of CoA thioesters in chlorogenic acid biosynthesis. We have investigated the enzymological aspects of the biosynthesis of chlorogenic acid in tomato fruits during the ripening phase.

RESULTS

Separation on DEAE-cellulose of extracts of tomato fruits showed an enzyme catalysing the synthesis of quinate esters from hydroxycinnamyl CoA derivatives and quinic acid (hydroxycinnamyl CoA:quinate: hydroxylcinnamyl transferase). This transferase was eluted in a position in the salt gradient corresponding to 0.065 M KCl. There is close correspondence in the elution position of the transferase activity in the synthesis of chlorogenic acid from caffeyl CoA and quinic acid (forward reaction) and in the cleavage of chlorogenic acid in the presence of CoA to form caffeyl CoA and quinic acid (reverse reaction). The same peak also shows activity in the corresponding forward and reverse reactions in the synthesis and breakdown of *p*-coumarylquinic acid.

Properties of transferase activity

An enzyme fraction partially purified (× 32) by $(NH_4)_2SO_4$ precipitation, desalting on Sephadex G25 and gradient elution from DEAE cellulose was used to study the properties of the transferase. This partially purified enzyme, which was free of thioesterase activity, can be stored at −20° for 2 months with only slight loss of activity. A spectrophotometric assay based on the difference in absorbance between the CoA thioesters and the quinate esters of caffeic and *p*-coumaric acids at 360 and 333 nm respectively was used in this work.

Table 1 shows that the esterification reactions are freely reversible. In the forward reaction, both *p*-coumaryl and caffeyl CoA form their corresponding quinate esters. In each case the reaction product has been shown to be the 5′ isomer. The activity towards *p*-coumaryl CoA is greater than that towards caffeyl CoA, and feruloyl CoA is also a substrate of the enzyme although the activity is only about a tenth of that with *p*-coumaryl CoA. The nature of the product with feruloyl CoA has not been investigated. The corresponding cinnamic acids will not

* The IUPAC system for numbering the quinic acid ring has been employed in this paper.

Table 1. Specificity of hydroxycinnamyl CoA:quinate hydroxycinnamyl transferase from tomato fruits

Forward reaction	Enzyme activity (nkat/mg protein)
Substrates	
p-coumaryl CoA	
+ quinic acid	28.0
+ shikimic acid	0.7
+ glucose	0
Caffeyl CoA	
+ quinic acid	16.2
+ shikimic acid	0.2
+ glucose	0
p-coumaric acid	
+ quinic acid	0
Caffeic acid	
+ quinic acid	0
Reverse reactions	
Substrates	
5′-*p*-coumarylquinic acid + CoA	48.2
4′-*p*-coumarylquinic acid + CoA	0.3
3′-*p*-coumarylquinic acid + CoA	0.2
Chlorogenic acid + CoA	22.1

replace their CoA thioesters in these reactions and glucose will not replace quinate. However, shikimic acid is a substrate for the enzyme but the rate of formation of the shikimate esters is less than 1/40th of that of the quinate esters. In the reverse reaction, the rate with *p*-coumarylquinic acid is roughly twice that with chlorogenic acid and the enzyme shows almost complete specificity for the 5′ isomers. The rates with the 3′ and 4′ isomers of *p*-coumarylquinic acid are only 0.6 and 0.4% respectively of the rate given by the 5′ isomer. Table 2 shows that in the forward reaction both the maximum velocity and the affinity (inversely proportional to the K_m value) are greater with *p*-coumaryl CoA compared with caffeyl CoA and the affinity for quinic acid is similar with either substrate but slightly greater with *p*-coumaryl CoA. In the reverse reaction, however, the maximum velocity with *p*-coumarylquinic acid is greater than that with chlorogenic acid but the affinity towards chlorogenic acid is slightly greater. The K_m value for CoA in the presence of chlorogenic acid is 0.084 mM.

The transferase shows a pH optimum of 7.0 for both the forward and reverse reactions and is unaffected by the presence of Mg^{2+}, BSA and DTE but is sensitive to the buffer system used in its assay. HEPES, Tris and ADA are all inhibitory compared with phosphate and cause between 35–45% inhibition. In some cases, the addition of EDTA partially relieves this inhibition of activity with an optimum effect at 1 mM. Even with phosphate, EDTA stimulates the rate by 10–15%. These buffer effects are shown in both the forward and reverse reactions as well as with either *p*-coumaryl or caffeyl derivatives.

Properties of ligase

Table 3 shows the substrate specificity of the *p*-coumarate CoA ligase of tomato fruit. The enzyme shows high activity towards *p*-coumaric and caffeic acids but none towards cinnamic acid. However, the affinity for *p*-coumaric acid is greater than that for caffeic acid. The enzyme also shows substantial activity towards ferulic, isoferulic and *m*-coumaric acids and lower activity with *p*-methoxy- and *m*-methoxycinnamic acids. Dimethoxycinnamic and sinapic acids are not activated. The ligase is eluted from the DEAE cellulose at 0.185 M KCl as a single peak of activity, and is completely separated from the transferase peak.

DISCUSSION

We have demonstrated with extracts of tomato fruits the enzymic potential for the conversion of *p*-coumaric acid to 5′-*p*-coumarylquinic acid and of caffeic acid to chlorogenic acid with the corresponding CoA thioesters as intermediates. These interconversions are catalysed by hydroxycinnamate CoA ligase and hydroxycinnamyl CoA quinate hydroxycinnamyl transferase, which have been separated completely by ion exchange chromatography. The ligase is similar in its general properties and substrate specificity to that of the enzyme described in potato tubers [9]. This tomato ligase does not exhibit the absolute requirement for free phenolic hydroxyl groups as shown by the enzymes from *Brassica* [10] and *Forsythia* [11] and differs in its pattern of activity towards minor substrates from the enzyme from either of these two sources and from the two isoenzymic forms

Table 2. Kinetic constants for the hydroxycinnamyl CoA:quinate hydroxycinnamyl transferase of tomato fruits

Substrate	Constants for	K_m (μM)	V_m (nkat/mg protein)
Forward reaction			
p-coumaryl CoA	*p*-coumaryl CoA	3	36.7
	quinic acid	430	33.6
caffeyl CoA	caffeyl CoA	29	20.0
	quinic acid	630	22.9
Reverse reaction			
5′-*p*-coumarylquinic acid	5′-*p*-coumarylquinic acid	92	39.8
Chlorogenic acid	Chlorogenic acid	60	28.1
	CoA	84	—

Table 3. Substrate specificity of tomato *p*-coumarate CoA ligase

Substrate	Enzyme activity in standard assay (substrate conc. = 0.5 mM) (pkats/mg protein)	K_m (μM)
Cinnamic acid	n.a.	—
p-coumaric acid	114	1.4
o-coumaric acid	15	—
m-coumaric acid	56	—
p-methoxycinnamic acid	10	—
m-methoxycinnamic acid	7	—
caffeic acid	88	8.9
ferulic acid	101	—
isoferulic acid	99	—
2,4-Dimethoxycinnamic acid	n.a.	—
Sinapic acid	n.a.	—

n.a.—Not activated.

found in *Glycine* [12]. The tomato ligase, in common with other plant ligases so far described shows a high affinity towards both *p*-coumaric and caffeic acids. However, it does not activate cinnamic acid.

The transferase activity is specific for the CoA thioesters of *p*-coumaric, caffeic and ferulic acids in the synthesis of their corresponding quinate esters. The corresponding cinnamic acids are not esterified. The activity and affinity are greater with *p*-coumaryl CoA than caffeyl CoA; ferulyl CoA is esterified only very slowly. The enzyme is specific for the formation of quinate esters; the corresponding reactions to form shikimate derivatives proceed at only 2% of the rate of formation of quinate esters. The enzyme is specific for the formation of the 5′ isomers of both *p*-coumarylquinic and chlorogenic acids.

The transferase reaction is reversible; both *p*-coumarylquinic and chlorogenic acids are cleaved in the presence of CoA to form the corresponding hydroxycinnamyl CoA esters and quinic acid. In this reverse reaction with *p*-coumarylquinic acid as substrate, the enzyme is specific for the 5′ isomer and the 3′ and 4′ isomers are cleaved at less than 1% of the rate with the 5′ isomer. In the reverse reaction the affinity of the enzyme for chlorogenic acid is slightly greater than that for *p*-coumarylquinic acid. Steck [7] has shown that labelled chlorogenic acid fed to tobacco tissue was metabolised and that a major product of its breakdown was caffeic acid. He also showed that it was metabolised to other phenolic compounds such as coumarins and lignins. The transferase activity in its reverse reaction could account for such interconversions with caffeyl CoA acting as a metabolic intermediate.

Evidence for the occurrence of *p*-coumarylquinic acid in higher plants has been described [13,14]. The substance isolated from immature apples was originally thought to be the 5′ isomer but more recent work [15] has shown that this is an artefact of the extraction procedure and the natural product is the 4′ isomer. Previously various workers [16,17] had shown that under alkaline conditions at temperatures above 50° [16] transesterification of various cinnamyl quinate esters occurs and interconversion of the 1′ ⇌ 3′ ⇌ 4′ ⇌ 5′ isomers was possible. Hanson [17] showed that such interconversions occurred on heating at 90° even at pH 7.0 and the particular buffer used had an important effect on the rate of isomerisation. This led us to investigate the stability of the *p*-coumaryl quinate ester substrates used in the present work. The 5′ isomer after 21 days low temperature storage in 0.1 M phosphate pH 7.0 at a concentration of 2 mM showed less than 2% isomerisation to the 4′ isomer. This was the longest period over which a substrate solution was used in the present series of experiments. Our experiments leave no doubt as to the specificity of the enzyme in the synthesis and breakdown of the 5′ isomers.

Stöckigt and Zenk [8] have presented evidence that caffeyl CoA is an intermediate in chlorogenic acid synthesis. We have separated two enzymes from tomatoes, one a ligase catalysing the formation of the CoA thioesters of hydroxycinnamic acids and the other a transferase catalysing the formation of quinate esters from the CoA thioesters. The transferase acts on *p*-coumaryl CoA and ferulyl CoA in addition to caffeyl CoA and in fact the rate of reaction and affinity for *p*-coumaryl CoA is greater than for caffeyl CoA. The ligase also shows greater affinity for substrate in the formation of *p*-coumaryl CoA than in the formation of caffeyl CoA. The ligase does not activate cinnamic acid and thus it is unlikely that cinnamyl CoA or cinnamylquinate is an intermediate in chlorogenic acid biosynthesis in the tomato and this agrees with the later work of Hanson [18]. The enzymic evidence suggests that two routes of chlorogenic acid biosynthesis are possible in tomato fruits via either *p*-coumaryl or caffeyl CoA. All the necessary enzymes are present: cinnamic acid 4-hydroxylase [19], *p*-coumarate and caffeate CoA ligases [10,11] *p*-coumarate hydroxylase [20] and *p*-coumarylquinic hydroxylase [3,4]. However, the specificity of the ligase and the transferase suggest that the route via *p*-coumaryl CoA and *p*-coumarylquinic acid is the more likely. This agrees with the labelling data of Steck [7].

There is increasing evidence that the CoA thioesters of cinnamic acids are important intermediates in the synthesis and breakdown of phenolic compounds [21–23]. The present results together with those of Stöckigt and Zenk [8] strongly suggest the CoA thioesters also have a role as intermediates in the esterification reaction leading to the formation of 5′-*p*-coumarylquinic acid and chlorogenic acid.

METHODS

Tomatoes (*Lycopersicon esculentum* var Eurocross BB) grown under glasshouse conditions at F.R.I., were picked at the breaker stage and stored until they reached the middle stage of ripening (the "orange" stage).

Extraction and purification of enzymes

150 g of the wall material of the tomatoes were homogenised in a stainless steel roller mill in 600 ml of a medium containing 0.1 M Tris pH 8.0, 0.25 M Sucrose, 1 mM EDTA, 1 mM DTE and 1% polyclar AT. The extract was clarified by centrifugation at 10000 *g* for 20 min and the supernatant made to 85% saturation with $(NH_4)_2SO_4$ and the resultant protein precipitate centrifuged at 40000 *g* for 30 min. The $(NH_4)_2SO_4$ ppt. was resuspended in 25 ml 0.005 M Tris–1 mM DTE pH 7.45

and desalted on a column of Sephadex G25 (30 × 2.5 cm) using the same buffer for elution of the protein fraction. The eluate from Sephadex was applied to a column of DEAE-cellulose (DE-52-14 × 1.5 cm). The protein fractions were eluted from the DEAE cellulose by a linear gradient of KCl in 0.005 M Tris–1 mM DTE pH 7.45 between 0 and 0.4 M KCl in a total volume of 650 ml. 3-ml fractions were collected and assayed for the various enzyme activities. The protein concentrations of these fractions were determined by the method of Lowry *et al.* [24] after precipitating with 20% TCA and redissolving the protein precipitate in 0.1 N NaOH.

Assay of enzyme activities

The *p*-coumarate CoA ligase activity was determined by the method previously described [10].

Hydroxycinnamyl CoA: quinate hydroxycinnamyl transferase

(i) *Synthesis of quinate esters.* A mixture, containing *p*-coumaryl CoA (16.5 nmol) or caffeyl CoA (16.8 nmol), Pi buffer pH 7.0 (100 μmol), EDTA (1 μmol) and enzyme in a final volume of 1 ml, was incubated at 30° and its absorbance at 333 nm (*p*-coumaryl CoA) or 360 nm (caffeyl CoA) was followed for a few min until it had reached a constant value. The reaction was initiated by the addition of quinic acid (2 μmol) to the test cell and an equal volume of Pi buffer to the control cell. The decrease in absorbance at the appropriate wavelength was followed for about 3 min. The rate of decrease in absorbance was linear for the first few min of the reaction and was used as a measure of the enzyme activity. Approximate extinction coefficients for the difference in absorbance between *p*-coumaryl CoA and *p*-coumarylquinic acid at 333 nm and between caffeyl CoA and chlorogenic acid at 360 nm were determined and these values (14000 and 11000 mmoles^{-1} cm^2 respectively) were used to convert the observed absorbance changes into rates of formation of the quinate esters. In the calculation to derive these values, the extinction coefficients for *p*-coumaryl and caffeyl CoA determined by Stöckigt and Zenk [25] were employed. The CoA thioesters were prepared using purified Swede *p*-hydroxycinnamate CoA ligase by the method previously described [22].

(ii) *Breakdown of quinate esters.* A mixture of chlorogenic acid or *p*-coumarylquinic acid (0.2 μmol), Pi pH 7.0 (100 μmol) and EDTA (1 μmol) and enzyme was incubated at 30° (1 ml final volume) and the absorbance at 360 nm (or 333 nm) followed as above. CoA (0.2 μmol) was added to the test cell and the increase in absorbance at the appropriate wavelength followed for a few min. The rates of change of absorbance which were linear over the first 5 min, were used in conjunction with the extinction coefficients shown above, to give rates of breakdown of the quinate esters.

Nature of the reaction products

In the experiments to determine the nature of the product in the synthetic reaction, after incubation the assay mixture (as shown above) was made acid with HOAc and extracted with EtOAc. The EtOAc phase was taken to dryness *in vacuo*, dissolved in EtOH and the product purified by chromatography. In the reaction between caffeyl CoA and quinic acid the product had the same R_f in 3 solvents (A, B, C) and gave the same UV spectrum under acid conditions and under alkaline conditions in the presence of Na BH_4 [26] as chlorogenic acid. The product after chromatography gave a positive reaction in the modified Hoepfner test [27] and on alkaline hydrolysis with N NaOH at 0° for 16 hr under N_2 yielded 2 products, one of which had had the same R_f in 3 solvents (A, B, C), the same UV spectra under acid and under alkaline conditions with $NaBH_4$ and gave the same pale green colouration on spraying with diazotised *o*-dianisidine as caffeic acid. The second hydrolysis product was non-aromatic in nature and had the same R_f in 3 solvents (A, B, C) as quinic acid and gave a characteristic colour reaction in each case with the Cartwright and Roberts reagent [28]. On this basis, it was concluded that the reaction product was chlorogenic acid. In the forward reaction between *p*-coumaryl CoA and quinic acid, the purified product had the same R_f as 5′-*p*-coumarylquinic acid in 2 solvents, (C, D) which both separate the 3′, 4′ and 5′ isomers of *p*-coumarylquinic acid. The R_f's of these 3′, 4′ and 5′ isomers are respectively 0.42, 0.58 and 0.63 in solvent C and 0.21, 0.35 and 0.52 in solvent D. The R_f of the reaction product is 0.63 in solvent C and 0.53 in solvent D. The product had the same UV spectrum under acid and alkaline conditions as *p*-coumarylquinic acid and gave a peach colouration with diazotised *o*-dianisidine. On alkaline hydrolysis the product yielded two compounds, one of which gave R_f values in solvents A and B, a colouration in the diazotised *o*-dianisidine test and acid and alkaline UV spectra identical to *p*-coumaric acid. The other hydrolysis product was shown to be identical in R_f to quinic acid in solvents A and B and gave a positive Cartwright and Robert's test. This evidence shows that the reaction product is 5′-*p*-coumarylquinic acid.

In the reverse reaction between chlorogenic acid and CoA the reaction mixture after incubation was evaporated to dryness *in vacuo* and the residue taken up in water and separated by PC in solvents A and B. The reaction products were shown to be quinic acid by its R_f in the two solvents and its characteristic colour reaction with the Cartwright and Robert's reagent and caffeyl CoA by its R_f in these two solvents and its UV spectrum. Similarly with *p*-coumarylquinic acid and CoA, the products were shown to be quinic acid and *p*-coumaryl CoA by their R_f's in solvents A and B, by the characteristic colour reaction for quinic acid and the characteristic UV spectrum for *p*-coumaryl CoA. Chromatography solvents used: A, BuOH–HOAc–H_2O (4:1:5) B, 5% HOAc, C, isobutyl methyl ketone–HCOOH–H_2O (3:1:2) D, $CHCl_3$–*t*-BuOH–HOAc–H_2O (85:15:15:30).

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