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FORMATION OF CoA ESTERS OF CINNAMIC ACID DERIVATIVES BY EXTRACTS OF *BRASSICA NAPO-BRASSICA* ROOT TISSUE

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Abstract—An enzyme fraction from aged swede root disks catalyses the formation of CoA thioesters of cinnamic acids in the presence of CoA, ATP and Mg^{2+}. The enzyme shows activity only to those cinnamic acid derivatives bearing a phenolic OH group, *p*-coumaric and ferulic acids being the most active substrates. The requirement for Mg^{2+} can be replaced by Mn^{2+}, Co^{2+} or Ni^{2+}. The requirement for ATP could not be replaced by GTP, CTP, UTP, ADP or AMP. ADP and AMP, but not pyrophosphate, inhibited the ATP dependent activation of *p*-coumarate. The activity was inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate which suggests a requirement for –SH groups for activation. The activity of the enzyme is low in freshly prepared disks but rises during ageing, particularly if the ageing is carried out in the presence of low concentrations of ethylene.

INTRODUCTION

THE OUTLINE of the pathway of phenolic acid and lignin biosynthesis is now well established.[1,2] There is still controversy as to whether the free acids or some activated derivatives are the intermediates in the pathway, but the enzymic evidence seems to suggest that the free acids as such are involved at several steps.[3–6] Hahlbrook and Grisebach[7] and Walton and Butt[8,9] have described enzymes from higher plants which will activate cinnamic acids in the presence of ATP and CoA to form CoA esters of the cinnamic acids but there is no direct evidence for the role these activated forms might play in phenolic metabolism.

In our previous studies on phenolic acid and lignin biosynthesis in swede root tissue[10,11] it was shown that the ethylene treatment stimulated an increased activity of phenylalanine ammonia lyase and increased formation of phenolic acid intermediates and lignin. In the

[1] NEISH, A. C. (1968) *Constitution and Biosynthesis of Lignin* (FREUDENBERG, K. and NEISH, A. C., eds.), Springer, Berlin.
[2] SARKANEN, K. V. (1971) *Lignins* (SARKANEN, K. V. and LUDWIG, C. H., eds.), pp. 95–112, Wiley–Interscience, London.
[3] KOUKOL, J. and CONN, E. E. (1961) *J. Biol. Chem.* **236,** 2692.
[4] RUSSELL, D. W. (1971) *J. Biol. Chem.* **246,** 3870.
[5] SATO, M. (1966) *Phytochemistry* **5,** 385.
[6] FINKLE, B. J. and NELSON, R. F. (1963) *Biochem. Biophys. Acta* **78,** 747.
[7] HAHLBROOK, K. and GRISEBACH, H. (1970) *FEBS Letters* **11,** 62.
[8] WALTON, E. and BUTT, V. S. (1970) *J. Exp. Botany* **21,** 887.
[9] WALTON, E. and BUTT, V. S. (1971) *Phytochemistry* **10,** 295.
[10] RHODES, M. J. C. and WOOLTORTON, L. S. C. (1971) *Phytochemistry* **10,** 1989.
[11] RHODES, M. J. C. and WOOLTORTON, L. S. C. (1973) *Phytochemistry* **12,** 107.

present work we have demonstrated the presence of an enzyme which catalyses the activation of phenolic acids and the stimulation of this activity by ethylene. The substrate specificity of the enzyme differs from those enzymes previously described in the literature.[7–9]

RESULTS

Two assay methods were used to follow the activation of phenolic acids by enzyme preparations from aged disks of swede roots. One was based on the formation of phenolic acid hydroxamates by reaction between the phenolic acyl CoA and hydroxylamine. The formation of hydroxamate was, in turn, estimated by spectrophotometric assay of the ferric chloride complexes of the hydroxamates[12] or by separation of the hydroxamate products from the substrate acids by PC and their estimation by UV spectrophotometry after elution from the paper. The second method of assay, which showed much greater sensitivity, was based on the differences in the spectral characteristics of cinnamic acids and their CoA esters at neutral pH in the range of 310–360 nm.[13] This second method (the direct spectrophotometric method) has been used for most of the experiments described here. In both assays, the pH optimum for activation with *p*-coumarate as substrate was 7·5.

TABLE 1. THE PROPERTIES OF *p*-COUMARYL CoA SYNTHETASE FROM SWEDE ROOT TISSUE

	Ferric chloride hydroxylamine assay (nmol/hr/mg protein)	Direct spectrophotometric assay (nmol/min/mg protein)
Complete system in air	45	20·2
in N_2	38	—
Complete system in air		
+ Boiled enzyme	0	—
− CoA	0	0
− ATP	0	0
− Mg^{2+}	13	3·7
− Coumarate	0	0

Table 1 shows the characteristics of the enzyme activity measured by both methods. It can be seen that the activation of *p*-coumaric acid requires a heat-labile factor which has an absolute requirement for ATP and CoA and a partial requirement for Mg^{2+}. The activity was not stimulated under anaerobic conditions and this is contrary to the findings of Walton and Butt with the cinnamyl CoA synthetase from spinach beet.[8,9] Table 2 shows that the K_m values of the enzyme for ATP and Mg^{2+} are of the same order, whereas

TABLE 2. THE APPARENT MICHAELIS CONSTANTS OF THE *p*-COUMARYL CoA SYNTHETASE

	K_m (mM)		
p-Coumarate	0·014	ATP*	0·19
Ferulate	0·031	Mg^{2+}*	0·21
		CoA*	0·026

* With *p*-coumarate as substrate

[12] JONES, M. E. and LIPMANN, F. (1955) *Methods Enzymol.* **1**, 585.
[13] GROSS, G. G. and ZENK, M. H. (1966) *Z. Naturforsch.* **21b**, 683.

that for *p*-coumarate is much lower. The enzyme shows a lower K_m and higher V_{max} (see Table 3) with *p*-coumarate as substrate compared with ferulate. Table 3 shows in greater detail the substrate specificity of the enzyme. The enzyme shows strong activity with *p*-coumarate, ferulate, isoferulate and caffeate as substrate and weaker activities towards *o*-coumaric *m*-coumaric and sinapic acids. None of the cinnamic acids without free phenolic OH groups tested were activated by the enzyme.

TABLE 3. SUBSTRATE SPECIFICITY OF *p*-COUMARYL CoA SYNTHETASE

Substrate	Enzymic activity (nmol activated/min/mg protein)	Substrate	Enzymic activity (nmol activated/min/mg protein)
Cinnamic acid	~0	Isoferulic	13·3
p-Coumaric	22	*p*-Methoxycinnamic	~0
o-Coumaric	2·4	3,4-Dimethoxycinnamic	~0
m-Coumaric	3·5	3,4,5-Trimethoxycinnamic	~0
Caffeic	15	Sinapic	0·4
Ferulic	16		

Table 4 shows the ability of certain divalent cations to replace the requirement for Mg^{2+} in the enzymic reaction. Mn^{2+}, Co^{2+} and, to a lesser extent, Ni^{2+} will replace the requirement for Mg^{2+}. Ca^{2+}, Cd^{2+} and Zn^{2+} will promote lower rates of activation but are much less efficient than either Mg^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+}. The K_m values for Mn^{2+} and Co^{2+} (0·23 mM in each case) are very similar to that for Mg^{2+} (0·21 mM). Table 4 also shows that Zn^{2+}, Cd^{2+} and Hg^{2+} inhibited the Mg^{2+} dependent rate in assays containing these cations in addition to Mg^{2+}. Hg^{2+} was the most inhibitory and at equimolar concentrations completely inhibited the Mg^{2+} dependent activity.

TABLE 4. THE SPECIFICITY OF THE *p*-COUMARYL CoA SYNTHETASE TOWARDS DIVALENT CATIONS

Cation (at 2·5 mM)	Activity relative to Mg^{2+} in promoting activation (Mg^{2+} activity = 100)	Inhibition of the Mg^{2+} dependent activity (%)	Cation (at 2·5 mM)	Activity relative to Mg^{2+} in promoting activation (Mg^{2+} activity = 100)	Inhibition of the Mg^{2+} dependent activity (%)
Be^{2+}	~0	~0	Zn^{2+}	28	22
Mg^{2+}	100	—	Cd^{2+}	20	80
Ca^{2+}	52	~0	Hg^{2+}	~0	100
Sr^{2+}	~0	~0	Mn^{2+}	100	—
Ba^{2+}	~0	~0	Co^{2+}	100	—
			Ni^{2+}	85	—

Table 5 shows that the requirement for ATP in the activation of *p*-coumarate could not be replaced by ADP, AMP, GTP, UTP or CTP. It also shows that while GTP, UTP and CTP had no effect on the ATP dependent activation, both ADP and AMP were inhibitory.

TABLE 5. THE ACTIVITY OF NUCLEOSIDE PHOSPHATE IN THE ACTIVATION OF *p*-COUMARATE

(mM)	Activity relative to ATP in promoting activation of *p*-coumarate (ATP dependent rate = 100)	Inhibitory effect on the ATP dependent rate (%)	(mM)	Activity relative to ATP in promoting activation of *p*-coumarate (ATP dependent rate = 100)	Inhibitory effect on the ATP dependent rate (%)
GTP 2·5	0	0	AMP 5·0	—	89
UTP 2·5	0	0	2·5	0	69·5
CTP 2·5	0	0	1·0	—	64
ADP 5·0	—	70	0·5	—	50
2·5	0	50	0·2	—	25
1·0	—	25	0·1	—	11
0·25	—	11·1			

AMP was the more inhibitory of the two, causing 70% inhibition at 2·5 mM. Table 6 shows the effect of various inhibitors on the formation of *p*-coumaryl CoA in the assay. The inhibition by *N*-ethyl maleimide and *p*-chloromercuribenzoate suggests the enzyme has a requirement for free –SH groups for its activity. NaF at 10^{-2} M had no effect on the activity and this would seem to suggest that ATPase was not a major problem in the assay. Pyrophosphate was not inhibitory, unlike AMP the other product of the hydrolysis of ATP during the activation of the *p*-coumarate. The effect of chelating agents on the activity is not clear cut. EDTA will inhibit at high concentrations but this inhibition is reversible by addition of Mg^{2+}. Neither Dieca nor *o*-phenanthroline (results not shown) was found to cause inhibition and this suggests there is a requirement for a divalent ion but not for another more firmly bound metal ion.

TABLE 6. THE EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF *p*-COUMARYL CoA SYNTHETASE

Inhibitor	Concn	% Inhibition of the activity	Inhibitor	Concn	% Inhibition of the activity
N-Ethylmaleimide	10^{-3} M	100	EDTA	10^{-2} M	82
	10^{-4} M	7		2×10^{-3} M	14
p-Chloromercuri-benzoate	10^{-3} M	100	DIECA	10^{-3} M	0
	10^{-4} M	50	Pyrophosphate	$2{\cdot}5\times10^{-3}$ M	0
	10^{-5} M	13·3	Sodium fluoride	10^{-2} M	3·3

Table 7 shows the activities towards *p*-coumarate and ferulate and of acetyl and malonyl CoA synthetases of crude extracts of disks taken immediately after cutting from swede roots or after a subsequent period of ageing in either air or air containing 8 ppm ethylene. The assays were done on extracts after ammonium sulphate precipitation and desalting on the Sephadex column by the $FeCl_3$–hydroxylamine method. Freshly prepared disks had a high capacity for the activation of acetate and malonate but very little activity towards the phenolic acid substrates. On ageing, the capacity to activate the phenolic acid substrates increased markedly, especially in the ethylene treated sample, whereas there was little change in the capacity for the activation of acetate and malonate.

TABLE 7. THE CHANGES IN PHENOLIC ACID, ACETYL AND MALONYL CoA SYNTHETASES DURING THE AGEING OF SWEDE ROOT DISKS

Substrate	(nmol formed/hr/mg protein) Freshly prepared disks	Disks aged for 20 hr in air	Disks aged for 20 hr in the presence of 8 ppm ethylene
p-Coumarate	4	19	44
Ferulate	0	9	26
Acetate	19	26	20
Malonate	11	16	8

DISCUSSION

The substrate specificity of the *p*-coumaryl CoA synthetase of swede roots differs markedly from that of other similar enzymes described in higher plants[7–9] and animals.[13] The swede enzyme is specific for the presence of phenolic OH groups in the substrate and is inactive with cinnamic acid and methoxylated cinnamic acid derivatives. It differs from the enzyme from spinach beet[8,9] and bovine liver mitochondria[13] which have the highest activity with cinnamic acid and very little activity with cinnamic acid derivatives bearing free OH groups. The enzyme from parsley[7] shows highest activity towards *p*-coumaric and ferulic acids but substantial activity with cinnamic acid and *p*-methoxycinnamic acid. There is at present no information as to whether there is a single enzyme of low specificity responsible for the observed activities or a number of enzymes of more limited specificity.

The demonstration of the capacity of plant tissues to make the CoA thioesters of substituted cinnamic acids prompts consideration of the role these compounds might play in the biosynthesis of cinnamic acids and of secondary plant products such as flavanoids and lignin. A role for *p*-coumaryl CoA in the formation of the A ring of flavanoids has been proposed by Hahlbrook *et al.*[14] The increased activity of the *p*-coumaryl CoA synthetase observed during the ageing of swede root disks in the presence of ethylene when increased phenolic acid and lignin biosynthesis has been shown to occur[11] suggests that this enzyme and its products, the CoA thioesters, might be involved in the biosynthesis of cinnamic acids and lignin. The possible involvement of CoA thioesters in lignin biosynthesis has been discussed by Zenk and Gross.[15] *In vivo* experiments have shown that higher plants can reduce ferulate to coniferaldehyde and coniferyl alcohol.[16] The reduction of cinnamic acids to their corresponding alcohols is probably a two-step reaction with the aldehydes as the intermediate. *In vitro*, the reduction of cinnamyl aldehydes to their alcohols, the immediate precursors of lignin, has been shown to be catalysed by an aromatic alcohol dehydrogenase.[17] Aldehyde dehydrogenases, which catalyse the conversion of aldehydes to acids, generally act irreversibly in the direction of aldehyde oxidation[18] but an acylating aldehyde dehydrogenase[19] does occur in certain microorganisms which catalyses a reversible reaction involving the CoA thioester of the acid substrate as an intermediate in the reduction.

[14] HAHLBROOK, K., EBEL, J., ORTMANN, R., SUTTER A., WELLMAN, E. and GRISEBACH, H. (1971) *Biochim. Biophys. Acta* **244**, 7.
[15] ZENK, M. H. and GROSS, G. G. (1972) *Recent Adv. Phytochem.* **4**, 87.
[16] HIGUCHI, T. and BROWN, S. A. (1963) *Can. J. Biochem. Physiol.* **41**, 621.
[17] DAVIES, D. D., UGOCHUKWU, E. N., PATIL, K. D. and TOWERS, G. H. N. (1973) *Phytochemistry* **12**, 531.
[18] JAKOBY, W. B. (1963) *Enzymes* **7**, 203.
[19] STADTMAN, E. R. and BURTON, R. M. (1955) *Methods Enzymol.* **1**, 518.

In an analogous situation it was shown that the reduction of fatty acids to their corresponding aldehydes involved CoA thioesters as intermediates.[20] In a preliminary communication, Zenk *et al.*[21] have demonstrated the presence of an enzyme system from a higher plant tissue which will catalyse the reduction of ferulic acid to coniferyl alcohol and evidence was presented which suggests that feruloyl CoA and coniferaldehyde were intermediates in the reaction. The substrate specificity of the CoA synthetase in swedes showing high activity towards *p*-coumarate and ferulate and low activity towards sinapate is consistent with a role for this enzyme in lignin biosynthesis since, as we have previously shown,[10,11] swede lignin is rich in coniferyl and *p*-hydroxyphenyl residues but has little or no syringyl residues. The reduction of cinnamic acids to their aldehydes and eventually their alcohols is an area in lignin biosynthesis in which there is very little information available and the role of CoA thioesters in this reduction is worthy of further study.

Many of the properties of the *p*-coumaryl CoA synthetase in swede roots are similar to those described for acetyl CoA synthetase[22] especially in relation to the specificity of the requirements for divalent metal ions and in the involvement of –SH groups in the enzyme activity. We have found, in agreement with Hahlbrook and Grisebach,[7] that the enzyme cannot be satisfactorily assayed at the stage of $(NH_4)_2SO_4$ precipitation as there appears to be an inhibitor present at this stage which is removed during the subsequent chromatography. We are at present studying the nature of this inhibitor and making attempts to purify the enzyme in order to study its properties in greater detail.

EXPERIMENTAL

Swede roots were harvested from plants grown in experimental plots at the Food Research Institute, Norwich and stored at 1° until required for analysis. The method of preparation of disks of root tissue and the conditions for ageing in the presence of ethylene were as previously described.[10,11]

Extraction and assay of the acyl CoA synthetase. 20 g of disks aged in the presence of 8 ppm ethylene for 20 hr were homogenized at 0° in an ultraturrax homogenizer for 1 min in 80 ml of a medium containing 0·1 M Tris pH 8·0, 1 mM EDTA, 0·25 M sucrose, 5 mM mercaptoethanol and 1% Polyclar AT. The homogenate was filtered through miracloth and the filtrate centrifuged at 55 000 *g* for 15 min at 0°. $(NH_4)_2SO_4$ was added to the supernatant to give 90% saturation and the protein precipitate separated by centrifugation at 55 000 *g* for 30 min at 0°. The protein fraction was resuspended in 5 ml 0·1 M Tris–2 mM mercaptoethanol pH 7·5 and applied to a column of Sephadex G25 (Medium grade, 1·5 × 20 cm) and eluted with the resuspension buffer. The protein fraction after Sephadex chromatography, which was yellow–brown in colour, was used for the assays using the $FeCl_3$–hydroxylamine assay.

The fraction after Sephadex chromatography was applied to a column of DEAE-cellulose (Whatman DE23, 1·5 × 12 cm) equilibrated with 0·1 M Tris pH 7·5 containing 2 mM mercaptoethanol. The fraction was washed on with a further sample of 0·1 M Tris pH 7·5 and the eluate discarded. The enzyme was recovered from the column by elution with 0·5 M KCl in 0·1 M Tris pH 7·5 containing 2 mM mercaptoethanol. This fraction was a pale straw colour and was used for all the assays involving the direct spectrophotometric method. At this stage in purification the enzyme is relatively stable, losing only 30% of its activity on storage at 0° for 7 days.

The assay mixture for the $FeCl_3$–hydroxylamine method included 50 μmol phosphate buffer pH 7·5, 1 μmol CoA, 5 μmol ATP, 5 μmol $MgCl_2$, 600 μmol hydroxylamine, 2 μmol substrate and 2–3 mg protein in a final vol. of 1·6 ml. The reaction mixture was incubated at 30° for 120 min and the formation of hydroxamate was shown to proceed linearly with time over 3 hr. The reaction was stopped by the addition of 0·4 ml of an acidic solution of $FeCl_3$,[7] the denatured protein was removed by centrifugation and the supernatant further clarified by filtration through glass fibre filter paper. The absorbancy of the $FeCl_3$ complex of the hydroxamate formed during the incubation was measured at 546 nm in a spectrophotometer against controls incubated in the absence of ATP and CoA and similarly treated. The extinction coefficients determined by Gross and Zenk[13] were used to calculate the yields of hydroxamates from the absorbance data.

[20] KOLATTUKUDY, P. E. (1970) *Biochemistry* **9,** 1095.
[21] MANSELL, R. L., STÖCKIGT, J. and ZENK, M. H. (1972) *Z. Pflanzenphysiol. Bd* **68S,** 286.
[22] WEBSTER, JR., L. T. (1969) *Methods Enzymol.* **8,** 375.
[23] LOWRY, O. H., ROSEBROUGH, N. J., FAU, A. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.

The product of the reaction with *p*-coumarate as substrate was studied by PC. After incubation the reaction was stopped by the addition of 8 vols. of acetone. The precipitated protein and salt were separated by centrifugation and the supernatant was evaporated to dryness *in vacuo*. The residue was taken up in a small vol. of acetone and applied to PCs and run in 2 solvents. It was shown that the product had the same R_f in the 2 solvents (I. Toluene–HCO_2Et–HCO_2H, 5:4:1 and II. iso-PrOH–NH_4OH–H_2O, 8:1:1), as an authentic sample of *p*-coumaryl hydroxamate prepared by the method of Hahlbrook and Grisebach.[7] The spots of the developed chromatogram, when viewed under UV light and then sprayed with 1% $FeCl_3$ solution, gave the same colours as the synthetic hydroxamates. The UV spectrum of the product and the visible spectrum of the product after spraying with $FeCl_3$ were the same as given by *p*-coumaryl hydroxamate and its ferric chloride complex respectively. Experiments using *p*-coumaric acid-[2-^{14}C] as the substrate of the enzyme showed that CoA and ATP stimulated the formation of a radioactive product which co-chromatographed with synthetic *p*-coumaryl hydroxamate in 2 solvents.

The assay mixture for the direct spectrophotometric assay was as follows: 100 μmol Tris buffer pH 7·5, 2·5 μmol $MgCl_2$, 2·5 μmol ATP, 0·5 μmol substrate and enzyme to a final vol. of 1 ml. This incubation mixture was set up in two 1-cm pathlength cells of the Cary 14 spectrophotometer and equilibrated at 30° until any small initial changes in extinction had ceased. Then 0.2 μmol of CoA was added to 1 cell and the increase in absorbance at 333 nm (with *p*-coumarate as substrate) due to the formation of CoA thioesters followed for 5 min at 30°. The rate of increase in absorbance is linear with time over this period and these changes in absorbance were used to calculate the rate of formation of CoA ester by applying the extinction coefficients determined by Gross and Zenk.[13] When substrates other than *p*-coumarate were treated, the wavelengths selected for following the reactions were the λ_{max} of the difference spectra between the particular cinnamic acid and its CoA esters determined by Gross and Zenk.[13] These all fall within the range 310–360 nm. With all substrates showing activity with the swede enzyme, difference spectra were determined after incubation and these agree closely with the published values. Attempts to isolate the product of incubation were made using the methods described in the literature[13] involving the use of low temp. PC using Whatman No. 3 paper and *n*-BuOH–HOAC–H_2O (5:2:3, upper) as the solvent when the CoA ester (R_f 0·50) separates well from the unreacted *p*-coumarate. Assay mixtures incubated in the presence of *p*-coumarate were shown to accumulate a product, the formation of which requires CoA, ATP and Mg^+ and which has the properties of *p*-coumaryl CoA as described in the literature. The product (R_f 0·5) gave the colour reaction with diazotized dianisidine characteristic of *p*-coumarate, after it had been subjected to alkaline hydrolysis by incubating the developed paper in an atmosphere of NH_3. On elution from the paper with 0·1 M phosphate pH 7·0 the product had a UV spectrum with an optimum at 333 nm. On alkaline hydrolysis (0·1 N NaOH–30 min at room temp.) and subsequent readjustment of the pH to 7·0 the peak at 333 nm was eliminated and a hydrolysis product with the neutral and alkaline spectra of *p*-coumaric acid was obtained.

Protein determinations were carried out on TCA precipitates of the enzyme fractions using the method of Lowry *et al.*[23]