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THE PHOSPHOHYDROLYSIS OF HYDROXYCINNAMOYL-COENZYME A THIOESTERS IN PLANT EXTRACTS

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Abstract—In barley seedling extracts, *p*-coumaroyl-CoA is rapidly hydrolysed to *p*-coumaroyl-dephospho-CoA, *p*-coumaroyl-4'-phosphopantetheine and *p*-coumaroyl-pantetheine *p*-Coumaroyl-4'-phosphopantetheine is active as a substrate of agmatine coumaroyl transferase in the formation of *p*-coumaroyl-agmatine, but *p*-coumaroyl-pantetheine is inactive The phosphohydrolysis can be partly inhibited by inorganic pyrophosphate, sodium fluoride and purine nucleotides A simplified method for the synthesis of *N*-hydroxysuccinimide esters of hydroxycinnamic acids, used in the synthesis of CoA thioesters, is also described

INTRODUCTION

The biosynthesis of *p*-coumaroylagmatine, an amide which occurs in barley seedlings together with its antifungal dimers, known as the hordatines [1, 2], is effected by a *p*-coumaroyl-CoA agmatine *N*-*p*-coumaroyl transferase (EC 2 3 1 —) [3] In the course of a study on the biosynthesis of *p*-coumaroylagmatine by this enzyme, *p*-coumaroyl-CoA was found to be rapidly hydrolysed in barley seedling extracts The CoA thioesters of hydroxycinnamic acids are fluorescent and it was therefore possible to use TLC to monitor their transformation during the enzymic reactions

Although cinnamoyl-CoA thioesters are very important intermediates in plant phenolic biochemistry [4], comparatively little is known about their metabolism The fate of cinnamoyl-CoA thioesters when they are mixed with barley extracts was therefore studied in order to optimize the formation of *p*-coumaroylagmatine from *p*-coumaroyl-CoA *in vitro* A simplified synthesis of *N*-hydroxysuccinimide esters of hydroxycinnamic acids, used in the synthesis of CoA thioesters, was also developed

RESULTS

When *p*-coumaroyl-CoA was incubated with a dialysed barley seedling extract, the original fluorescent spot due to *p*-coumaroyl-CoA disappeared and simultaneously new products with higher R_f and similar fluorescence were detected Depending on the incubation time, up to three new fluorescent spots (A, B and C) could be found The time course of these reactions is shown in Fig 1 Three products could also be obtained from either caffeoyl-, feruloyl- or sinapoyl-CoA thioesters after incubation with the barley seedling extract No hydrolysis occurred without the plant extract When extracts boiled for 1 min were used, B and C could not be detected, but A was still formed at *ca* 50% of the rate in the unboiled extract Residual activity was still detectable after 15 min incubation at 100° The pH of the incubation medium was not critical, the hydrolysis occurring over a wide range of pH, with an optimum between 8 and 9

Identification of A, B and C

The kinetic study strongly indicated the following sequence *p*-coumaroyl-CoA → A → B → C When A was purified by TLC and incubated with the enzyme extract, only B and C could be found Similarly, C was the only product formed from B Hydroxycinnamoyl-CoA thio-

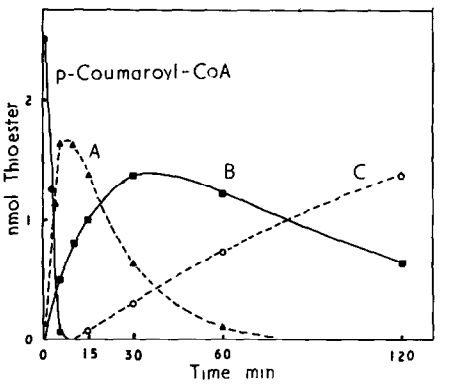

Fig 1 *p*-Coumaroyl-CoA (75 nmol, 75 μl) (—●—) was incubated with the enzyme extract (150 μl) at 30° The reaction was stopped by adding 10 μl acetic acid to 20 μl aliquots and 10 μl samples were applied to a TLC plate which was run in solvent 1 The products A (- -▲- -), B (—■—) and C (- -○- -) were quantified by fluorescence (see Experimental) See Fig 2 for structures

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Table 1 Properties and identification of the hydrolysis products of *p*-coumaroyl-CoA

	UV 260 nm	UV 333 nm	Alkaline hydrolysis	Hydroxyl-aminolysis	Mol Pi/mol thioester*	TLC R_f Solvent 1	TLC R_f Solvent 2	Activity in the transferase assay
p-Coumaroyl-CoA	+	+	+	+	3 24 (3)	0 40	0 60	+
p-Coumaroyl-dephospho-CoA (A)	+	+	+	+	1 86 (2)	0 48	0 66	+
p-Coumaroyl-4'-phosphopantetheine (B)	−	+	+	+	1 24 (1)	0 55	0 76	+
p-Coumaroyl-pantetheine (C)	−	+	+	+	0 19 (0)	0 88	0 91	−

*The actual value obtained is shown, together with the theoretical value in parentheses

esters show a characteristic UV spectrum with two peaks, one at 260 nm (adenine) and another between 333 and 352 nm (thioester bond) The hydroxycinnamoyl-CoA thioesters also undergo alkaline hydrolysis and hydroxyl-aminolysis at pH 7 5 [5] These properties, together with phosphate determination after hydrolysis with alkaline phosphatase and reaction in the *p*-coumaroyl-CoA agmatine *N*-*p*-coumaroyl transferase assay, were used to identify A, B and C (Table 1) A, B and C were also co-chromatographed in solvents 1 and 2 with the hydrolysis products obtained from *p*-coumaroyl-CoA in the presence of alkaline phosphatase The fact that the *p*-coumaroyl moiety was not modified in the reaction was further confirmed by reduction of the thioesters with sodium borohydride in water *p*-Coumaroyl-CoA, A, B and C gave *p*-hydroxyphenylpropanol as determined by their mass spectra Neither AMP nor adenosine could be easily detected on TLC as a product of the reaction, due to a rapid hydrolysis to adenine The complete pathway is presented in Fig 2

Effect of inhibitors

The complete phosphohydrolysis of *p*-coumaroyl-CoA

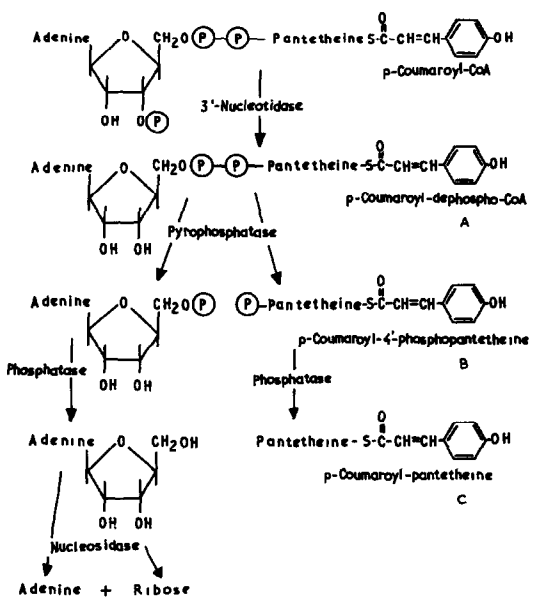

Fig 2 Phosphohydrolysis pathway of *p*-coumaroyl-CoA by crude barley extracts

involves several steps which could be catalysed by the enzymes 3'-nucleotidase, pyrophosphatase and a monophosphatase Inorganic phosphate and sodium fluoride [6] are often used as inhibitors of phosphatases Plant nucleases, which contain phosphodiesterase and 3'-nucleotidase activities, are inhibited by adenine nucleotides [7] We therefore also tested ATP, ADP and AMP as inhibitors The results are summarized in Table 2 Inorganic phosphate (50 mM) had very little effect on the formation of A and B but completely inhibited the hydrolysis of B to C

Phosphohydrolysis of hydroxycinnamoyl-CoA thioesters in other plants

Similar results were obtained using extracts from oat, wheat, maize and pea seedlings Extracts of mature tobacco leaves also catalysed the same reactions

DISCUSSION

The chemical synthesis of hydroxycinnamoyl-CoA thioesters is complicated by the reactivity of the phenolic hydroxyl groups during activation of the carboxylic acid Chemical protection and deprotection of the hydroxyl group can lead to the hydrolysis of the thioester bond [5] *N*-Hydroxysuccinimide esters of hydroxycinnamic acids obtained using dicyclohexylcarbodiimide (DCC) as coupling agent, without protection of the hydroxyl groups, must therefore be purified from a mixture of compounds, and the yield can be as low as 15% in the case of caffeic acid [5] We found, however, that it is possible to simplify the chemical synthesis using pyridine as solvent during the coupling reaction of the hydroxycinnamic acid to the *N*-hydroxysuccinimide (see Experimental) In a basic solvent, phenolic hydroxyl groups show decreased reactivity, as the activation of DCC depends on proton availability The *N*-hydroxysuccinimide esters obtained by this method are sufficiently pure to be used in the transesterification reaction with CoA without further purification The yield from CoA was *ca* 40% for cinnamoyl-, feruloyl- and sinapoyl-CoA and *ca* 30% for caffeoyl-CoA The basicity of the solvent in the coupling reaction was found to be an important parameter in the synthesis of *O*-peptides involving the hydroxyl groups of tyrosine, serine and threonine [8]

The fluorescence of the hydroxycinnamic acids allowed us to detect the rapid phosphohydrolysis of the hydroxycinnamoyl-CoA thioesters Barley is known to contain 3'-nucleotidase activity and the enzyme from this plant has been used in the identification of CoA [9, 10] As observed in ref [9], this enzyme is often remarkably heat-

Table 2 Effect of different inhibitors on the phosphohydrolysis of *p*-coumaroyl-CoA

	Control	Pi		PPi		NAF		AMP		ADP		ATP	
		5 mM	50 mM	5 mM	50 mM	5 mM	50 mM	5 mM	50 mM	5 mM	50 mM	5 mM	50 mM
p-Coumaroyl-CoA	0	0	0	0	34	0	0	0	0	0	27	0	21
p-Coumaroyl-dephospho-CoA (A)	29	26	30	35	49	39	79	79	100	73	68	69	51
p-Coumaroyl-4′-phosphopantetheine (B)	71	74	70	65	17	61	21	21	0	27	5	32	28

p-Coumaroyl-CoA (25 nmol, 25 μl) was incubated with a dialysed barley extract (50 μl) without (control) or with different inhibitors (25 μl) for 15 min in 0 1 M Tris–HCl buffer, pH 8 The reaction was stopped with acetic acid and the volume corresponding to 2 5 nmol thioester was applied to a TLC plate and run in solvent 1 Results are expressed in percentage of fluorescence recovered in the different products Negligible amounts of *p*-coumaroylpantetheine (C) could be detected in these conditions

stable and has been found in a number of plants After incubation of the barley extract at 100° for 1 min, the reaction could therefore be stopped at the first step (A) The inhibition by inorganic phosphate of the hydrolysis of *p*-coumaroyl-4′-phosphopantetheine was used to stop the reaction sequence at B

A number of enzymes could be involved in the observed hydrolysis Nucleases which exhibit both phosphodiesterase and 3′-nucleotidase activities are known to occur in plants and have been characterized in barley [11, 12] Nucleotide pyrophosphatases are widespread in animal tissues [13] and in plants [14, 15] The elucidation of the complete pathway was complicated by the occurrence of an active nucleosidase, already characterized in barley [16] Neither AMP nor adenosine was present in detectable amounts after incubation with the barley extract, but adenine could be detected with the Wood reagent and had R_f values in solvents 1 and 2 similar to those of *p*-coumaroyl-4′-phosphopantetheine, and sometimes confused the UV spectrum

Specific phosphatases hydrolysing CoA have been characterized in animals [17–19] However, the hydrolysis observed in a crude plant extract is very likely to be unspecific Acyl-CoA thioesters of fatty acids have been extensively used in research on lipid metabolism *In vitro* formation of acyl-pantetheine from acyl-CoA by liver plasma membranes has been reported [20]

It is very likely that the phosphohydrolysis of acyl-CoA thioesters in crude plant extracts is common Coenzyme A itself is known to be hydrolysed by the widespread 3′-nucleotidase [9] This hydrolysis could interfere with other reactions when CoA is used as acyl acceptor or donor Dephospho-CoA, 4′-phosphopantetheine and pantetheine are known to be used as substrates by acyl-CoA synthetase from rat liver [21] and *Candida lipolytica* [22] although they are less active than CoA In the oxidative decarboxylation of α-ketoglutarate, dephospho-CoA was found to be active at high concentration but phosphopantetheine was inactive [23] The transacetylase from *E coli* uses only intact CoA [24] and the sulphanilamide assay is known to use fragments of CoA [24] Covalently linked 4′-phosphopantetheine is used by acyl carrier proteins We found that *p*-coumaroyl-dephospho-CoA and *p*-coumaroyl-4′-phosphopantetheine were used as substrates in the formation of *p*-coumaroylagmatine No activity was detected with *p*-coumaroylpantetheine Separation of the agmatine coumaroyl transferase from the different phosphatases in order to determine the relative activity of *p*-coumaroyl-CoA, A and B was not attempted, in view of the difficulty of obtaining a phosphatase-free agmatine coumaroyltransferase activity

Among the inhibitors tested, those with a pyrophosphate bond were the most active and even inhibited the 3′-nucleotidase, possibly indicating that only one enzyme is involved in the first two steps, although the two activities can be differentiated on the basis of heat stability Inorganic pyrophosphatase is an effective inhibitor and did not inhibit the agmatine coumaroyltransferase It should therefore be useful in the detection of weak transferase activities, when long incubation times are needed

An alternative to the use of inhibitors would be the chromatographic separation of the enzyme involved Affinity chromatography on concanavalin A has been found to be very effective in the adsorption of nucleases from plant extracts [12] and could be used in the preparation of nuclease-free enzymic extracts if intact CoA is essential to detect an enzymic activity

EXPERIMENTAL

Barley (*Hordeum vulgare* L cv Proctor) was grown as previously described [3]

TLC was on cellulose CC41 using solvents—1 *n*-BuOH–EtOH–H_2O (4 1 2) (R_fs AMP, 0 25, adenosine, 0 48, adenine, 0 52, *p*-coumaroylagmatine, 0 63), 2 *n*-BuOH–HOAc–H_2O (5 2 3) (AMP, 0 31, adenosine, 0 66, adenine, 0 66), 3 *n*-BuOH–HOAc–H_2O (4 1 5, upper) (*p*-coumaroylagmatine, 0 77) See Table 1 for R_fs of *p*-coumaroyl-CoA, A, B and C

[G-^{14}C]-*Agmatine* was prepared as described in ref [3] and radioactive spots were visualized using a Betagraph

N-*Hydroxysuccinimide esters* were prepared as follows *p*-Coumaric acid (1 64 g, 10 mmol) and *N*-hydroxysuccinimide (1 15 g, 10 mmol) were dissolved in cold, anhydrous pyridine (20 ml) Dicyclohexylcarbodiimide (2 26 g, 11 mmol) was added, and the mixture was stirred at 4° in the dark After 24 hr, the pyridine was evapd to dryness at 40° under red pres and the residue dissolved in EtOAc The dicyclohexylurea was filtered off and the filtrate extracted with an equal vol of cold 1 M $NaHCO_3$ The EtOAc was then evapd and the *N*-hydroxysuccinimide ester was crystallized from EtOAc–petrol (bp 60–80°) (2 1) *N*-Hydroxysuccinimide esters of ferulic and sinapic acids were prepared in a similar way For caffeic acid, the coupling reaction was done under N_2 The purity of the esters was assayed by TLC on Kieselgel HF_{254} with $CHCl_3$–MeOH (20 1) as solvent [5] and by ^{1}H NMR

Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters as described in ref [5]

Enzyme preparation 3-Day-old barley seedlings were homogenized with a pestle and mortar with sand in 2 vols 0 1 M Tris–HCl buffer (pH 8 5) The extract was centrifuged at 20 000 *g* for 20 min and the supernatant dialysed for 16 hr in 0 01 M Tris buffer, pH 8 5

Preparation and purification of A, B and C *p*-Coumaroyl-dephospho-CoA (A) The enzyme extract (1 ml) was incubated at 100° for 1 min and after cooling to 30° *p*-coumaroyl-CoA (0 2 μmol, 200 μl) was added The mixture was incubated at 30° for 15 min and the reaction stopped with HOAc (100 μl, 17 M) After precipitation, the proteins were removed by centrifugation and the supernatant was applied to prep TLC plates and chromatographed in solvent 1

p-Coumaroyl-4'-phosphopantetheine (B) *p*-Coumaroyl-CoA (0 2 μmol) was incubated with 1 ml of enzyme extract in 0 05 M NaPi buffer (pH 8) for 1 hr B was purified in solvent 2

p-Coumaroyl-pantetheine (C) *p*-Coumaroyl-CoA (0 2 μmol) was incubated with 1 ml of enzyme extract for 2 hr in Tris buffer (pH 8 5) After acidification, C could be extracted in Et_2O C was purified in solvent 1 A, B and C were eluted from the TLC cellulose with H_2O and freeze-dried

Inorganic phosphate was assayed using the method of ref [25] Alkaline phosphatase from bovine intestine (Sigma) containing 12% pyrophosphatase activity was used to assay the total Pi The samples were incubated in Tris buffer (0 01 M, pH 9) with 5 units of alkaline phosphatase for 2 hr at 30° Only C was obtained from coumaroyl-CoA in these conditions but A and B could be obtained using non-optimal conditions (pH 7, dilute enzyme)

All the thioesters of hydroxycinnamic acid were quantified in soln using the extinction coefficients determined in ref [5] UV spectra were recorded in 0 1 M NaPi buffer (pH 7) At pH 7, A had the same UV spectrum as *p*-coumaroyl-CoA Only minor differences could be detected between their spectra at pH 9 B and C had the same spectra with λ_{max} 333 nm and no peak at 260 nm The same extinction coefficient (21×10^6 cm^2/mol) was used for *p*-coumaroyl-CoA, A, B and C only a slight increase (10%) in absorbance could be detected during the incubation of *p*-coumaroyl-CoA with alkaline phosphatase, indicating that the adenine and the coumaric acid parts do not interact strongly in the UV absorbance spectrum

The thioesters of hydroxycinnamic acids were detected on TLC by fluorescence after spraying the plates with a mixture of triethanolamine in *iso*-PrOH (1 4) The fluorescence was quantified using a Vitatron densitometer by excitation at 365 nm and emission at 507 nm A known amount of *p*-coumaroyl-CoA was spotted on the TLC plate and used as a standard

AMP, adenosine and adenine were detected on the TLC plates after chromatography, using the Wood reagent

p-Coumaroyl-CoA agmatine N-p-coumaroyl transferase assay 50 μl of the crude enzyme extract (after centrifugation) was incubated with the thioester (10 nmol, 10 μl) and 25 μl [^{14}C]agmatine (1 μCi/ml) for 1 hr at 30° *p*-Coumaroylagmatine was identified by co-chromatography in solvents 1 and 3, and electrophoresis on a cellulose plate (400 V, 1 hr, pyridine–HOAc–H_2O, 1 5 94)

$NaBH_4$ *reduction* of the thioesters was performed as described in ref [26] The products were extracted in Et_2O The EIMS [probe, 70 eV, *m/z* (rel int) 152 $[M]^+$ (32), 134 (15), 133 (18), 108 (15), 107 (100), 77 (15)], obtained from the reduction product of *p*-coumaroyl-CoA indicated that the thioester and the olefinic double bond were reduced, leading to 3-(*p*-hydroxyphenyl)-1-propanol The same fragments could be found in the MS of the reduction products of A, B and C

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