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MULTIPLE FORMS OF HYDROXYCINNAMATE : CoA LIGASE IN ETIOLATED PEA SEEDLINGS

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Key Word Index—*Pisum sativum*; Leguminosae; pea shoots; phenylpropanoid biosynthesis; hydroxycinnamate CoA ligase; multiple forms.

Abstract—A survey of a range of plant tissues showed that the hydroxycinnamate CoA ligase in crude extracts of pea shoots had a high relative activity towards sinapic and other methoxycinnamic acids, together with high activity with *p*-coumaric acid. The pea enzyme has been resolved by chromatography on DEAE-cellulose into two peaks which differ in their substrate specificity. The form which elutes at relatively low salt concentrations has a ratio activity towards *p*-coumaric and sinapic acids of about 1.8:1 while the form eluting at higher salt concentrations, although showing very high activity with *p*-coumaric acid, is inactive towards sinapic acid. The pattern of elution of these forms following gel filtration on Ultragel AcA 34 and Sephadex G100 suggests that these two isoenzymes which differ in ionic properties and substrate specificity can exist in two or three molecular weight forms and there is evidence that these forms are under certain circumstances interconvertible.

INTRODUCTION

The CoA thioesters of substituted cinnamic acids have been shown to be important intermediates in the biosynthesis of lignin, flavonoids and chlorogenic acid in higher plants [1–3]. The biosynthesis of lignin involves the reduction of free cinnamic acids to their corresponding alcohols via the CoA thioesters and the aldehydes, the reduction being catalysed by three enzymes, hydroxycinnamate : CoA ligase, hydroxycinnamyl CoA reductase and NADP specific cinnamyl alcohol dehydrogenase [4, 5].

The first of these enzymes, hydroxycinnamate CoA ligase, is involved in the synthesis of chlorogenic acid [3, 6] and flavonoids [7] as well as in lignin biosynthesis. It has been studied in a number of plant tissues and the existence of two isoenzymes has been shown in studies using extracts of cell suspension cultures of soyabean [8] and of *Petunia* leaves [9]. These isoenzymes differ in their activity towards sinapic acid and other methoxylated cinnamic acids [8]. 'Ligase I' which has a high affinity for sinapic acid and other methoxylated cinnamic acids was thought to be specifically involved in lignin biosynthesis while the ligase II which shows no activity towards sinapic acid was thought to be involved in flavonoid biosynthesis [8]. However, in other work lignification was correlated with the presence of a ligase II isoenzyme in the absence of ligase I [10, 11]. Thus it is possible that both isoenzymes may be involved in lignification in particular tissues and here the role of ligase I may be in the reduction of sinapic acid to supply syringyl residues for the lignin biosynthesis.

The present paper describes a survey of a range of plant tissues in which the substrate specificities of the ligase enzyme were studied in order to select species relatively rich in 'ligase I'. The separation and properties of the ligase enzymes isolated from etiolated pea seedlings is then described and evidence that this enzyme exists in the tissue in multiple forms differing in ionic properties, MW and in substrate specificity is presented.

RESULTS

A preliminary survey of the substrate specificity of the ligase was carried out using a large variety of plant materials as a source of enzyme. The enzyme fraction used in each case was obtained after $(NH_4)_2SO_4$ precipitation (0–80% saturation), gel filtration on Sephadex G-25 Medium and bulk elution of DEAE-cellulose (DE23) with 0.5 M KCl. It can be seen from Table 1 that the hydroxycinnamate CoA ligase is widely distributed. Activity towards sinapic acid was found in extracts of runner bean, soyabean, pea shoots and leeks, but was absent in extracts of aubergine, marrow and peppers. The three leguminous species combined high total ligase activity with relatively high activity towards sinapic acid and of these three species the pea shoot was selected for further study.

Extracts of etiolated pea shoots were fractionated by $(NH_4)_2SO_4$. The 50–75% fraction was desalted on Sephadex G25 and the desalted fraction separated on a column of DEAE cellulose using a linear gradient of salt between 0–0.5 M KCl in 0.005 M Tris pH 7.45 containing 1mM DTE. Fig. 1 shows that two distinct peaks of hydroxycinnamate : CoA ligase (I and II) were eluted at salt concentrations of 0.12 and 0.2 M KCl respectively. The smaller peak (I) had a relatively high activity towards sinapic acid, the ratio of *p*-coumaric : sinapic acid being 1.8:1, whereas peak II had virtually no sinapate: CoA ligase activity.

Table 2 shows further details of the substrate specificity of the two ligase isoenzymes. Both isoenzymes show high

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Table 1. Substrate specificity of hydroxycinnamate : CoA ligase from various plant tissues

Enzyme source	Total ligase activity with *p*-coumaric acid as substrate (nmol/min/50 g tissue)	Activity relative to *p*-coumaric acid = 100					
		ferulic acid	isoferulic acid	caffeic acid	sinapic acid	*p*-methoxy cinnamic acid	cinnamic acid
Runner bean	26.7	54	48	81	28	15	nd
Soybean shoots	180	38	65	14	22	nd	6
Pea shoots	62.9	52	38	36	31	3.8	nd
*Aged disks of cucumber	90.7	51	15	21	1.4	nd	0.4
*Aged disks of marrow	21.9	16	20	13	nd	nd	nd
Aubergine	12.3	102	130	nd	nd	12	nd
Green pepper	4.4	nd	48	32	nd	nd	nd
Red pepper	2.8	57	75	nd	nd	nd	nd
Leek	7.0	106	69	34	27	14	nd

nd = No activity detected. No activity was detected in extracts of apple, avocado, cabbage or melon and low total activities of 1.9 and 4.5 nmol/min/50 g tissue were found respectively in extracts of celery and cauliflower florets.

*Disks (10 × 2 mm) of the flesh of cucumber or marrow were prepared and aged in the medium and under the conditions previously described for swede root disks [10]. This ageing procedure was shown to increase the ligase which was almost undetectable in freshly prepared disks.

activity towards *p*-coumaric, ferulic, isoferulic and caffeic acids and the relative activities towards these four acids is approximately the same for both forms of the enzymes. However peak I shows high activity towards sinapic, *p*-methoxycinnamic and 3,4-dimethoxycinnamic acids while peak II is almost inactive towards these substrates. The ratios of activity of *p*-coumaric compared with sinapic acid and 3,4-dimethoxycinnamic acid is 0.56 and 0.85 respectively for peak I compared with only 0.02 and 0.03 for peak II. The slight activity with these methoxylated substrates in peak II could be due in part to incomplete separation between the two forms of the enzyme on the DEAE cellulose column.

The 50–75% ammonium sulphate fraction was also applied to a column of hydroxyapatite (Bio-gel HT) and at least three peaks of activity were observed following elution of the column with a linear gradient between 0.01–0.2 M KH_2PO_4 buffer pH 6.8. Both the major peaks after hydroxyapatite chromatography showed activity towards both *p*-coumaric and sinapic acids. The minor peak had activity only towards *p*-coumaric acid. When the same ammonium sulphate fraction was applied after desalting to a column of CM cellulose the activity of hydroxycinnamate CoA ligase was not retained.

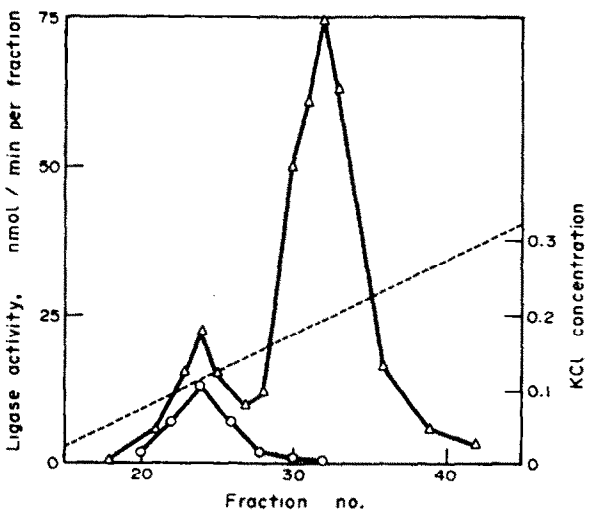

Fig. 1. Separation of pea hydroxycinnamate CoA ligase on DEAE cellulose. Activity with both *p*-coumaric acid (Δ–Δ) and sinapic acid (O–O) as substrate was measured.

Fig. 2a shows the pattern of elution when the 50–75% $(NH_4)_2SO_4$ fraction was applied to a column of Ultragel AcA 34 and eluted with 0.2 M Tris pH 7.45 containing 1mM DTE. The Ultragel column was calibrated for MW by determining the elution volumes of proteins of known MW.

The pea ligase gave three peaks of activity following chromatography on Ultragel with a major peak (UI) of MW 75 000 followed by two smaller peaks (UII) having MW's less than 20 000. The same pattern of elution was obtained using either *p*-coumaric or sinapic acid as substrate. The ratio of activity with *p*-coumaric and to that with sinapic acid was about 7–8 : 1 over the whole elution pattern, approximately the same as for the crude $(NH_4)_2SO_4$ fraction. The MWs obtained from the elution

Table 2. Substrate specificities of the hydroxycinnamate : CoA ligase isoenzymes of pea shoots separated by chromatography on DEAE-cellulose

Substrate	Enzyme activity			
	nmol/min/10 g fr. wt		Relative to *p*-coumaric acid	
	peak I	peak II	peak I	peak II
p-Coumaric acid	2.72	9.0	100	100
Ferulic acid	2.12	5.7	78	63
Isoferulic acid	1.53	4.65	56	52
p-Methoxycinnamic acid	0.39	0.14	14	2
Sinapic acid	1.55	0.19	56	2
3,4-Dimethoxycinnamic acid	2.30	0.26	85	3
Caffeic acid	1.04	2.43	38	27
Cinnamic acid	0	1.04	0	12

pattern on Ultragel columns were confirmed by repeating the separation of the $(NH_4)_2SO_4$ fraction on a column of Sephadex G100 calibrated for MW in the usual way. For further work on the high and low MW forms after Ultragel chromatography, tubes of the first peak were combined as the high MW form (UI) while the two subsequent peaks were combined as the low MW form (UII).

Both forms of the enzyme were found to have similar properties and showed dependence on CoA, ATP and Mg^{2+}. The same is true of the two forms separated on DEAE cellulose which were shown to have the same pH optimum at pH 7.5 (the pH optimum and the pH's for half maximal activity at 6.1 and 8.4 were determined using Tris and phosphate buffers in the range 5.5–8.5). In peak I the same pH optimum was obtained whether *p*-coumaric or sinapic acid was used as substrate.

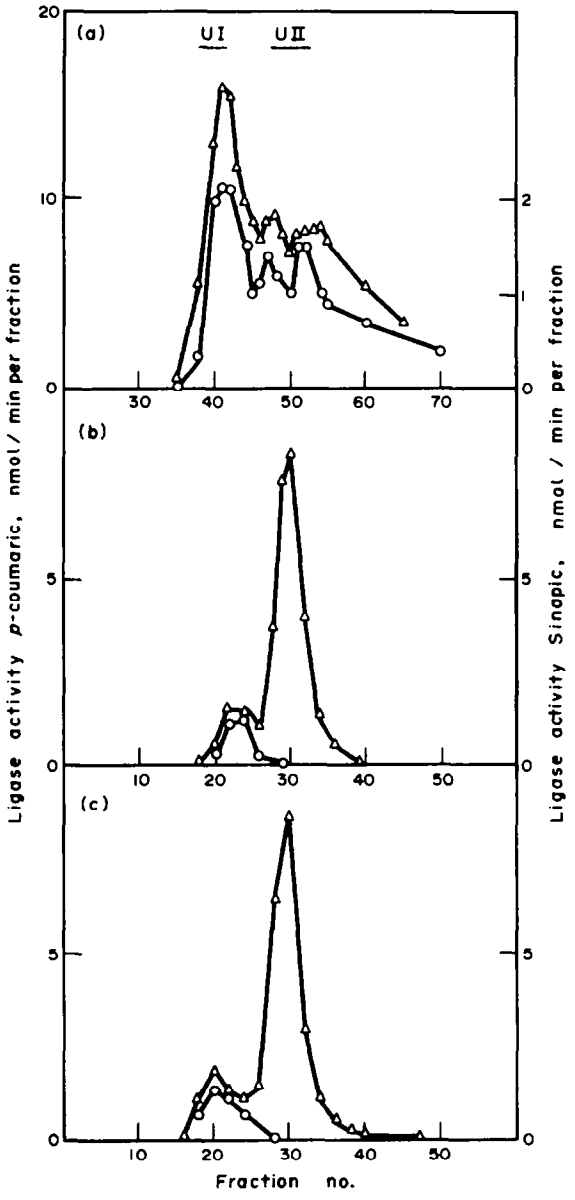

Fig. 2. a. The separation of pea hydroxycinnamate CoA ligase on Ultragel AcA 34. The activity was measured using either *p*-coumaric acid (△–△) or sinapic acid (○–○) as substrate. After chromatography the high and low molecular weight fractions (I and II) were combined, concentrated with $(NH_4)_2SO_4$ desalted on Sephadex G25 and applied to DEAE-cellulose under the standard conditions. The separations obtained with the high (Fig. 2b) and low (Fig. 2c) molecular weight forms on DEAE-cellulose are shown in the lower part of the figure.

Table 3. Substrate specificities of the high and low MW forms of the hydroxycinnamate CoA ligase of pea shoots separated on Ultragel AcA 34

Substrate	Enzyme activity			
	nmol/min/10 g fr. wt		Relative to *p*-coumaric acid	
	UI	UII	UI	UII
p-Coumaric acid	2.52	0.66	100	100
Ferulic acid	1.77	0.45	70	68
Isoferulic acid	1.35	0.23	54	35
p-Methoxycinnamic acid	0.14	0	5	0
Sinapic acid	0.47	0.29	19	44
3,4-Dimethoxycinnamic acid	0.68	0.33	27	50
Caffeic acid	0.89	0.25	35	38
Cinnamic acid	0.10	0	4	0

The high and low MW peaks from Ultragel were applied to DEAE-cellulose and eluted under the standard conditions with the linear gradient of KCl. In both cases the elution patterns obtained with the high (Fig. 2b) and low (Fig. 2c) MW fractions were identical to each other and to that obtained when the original $(NH_4)_2SO_4$ fraction was applied directly to DEAE-cellulose (see Fig. 1). Both forms gave two peaks on DEAE-cellulose, a minor one eluting at about 0.12 M KCl and a major one eluting at 0.2 M KCl. The minor one was active with both *p*-coumaric and sinapic acids while the major peak was specific for *p*-coumaric acid. Evidence that the two forms separated on Ultragel are interconvertible was obtained by rechromatography of the low MW form on the Ultragel column. Here the low MW peak gave rise to ligase eluting in the high MW position indicating that the low can be converted to the high MW form.

Table 3 shows the substrate specificities of the two forms of the enzyme following Ultragel AcA 34 chromatography. Both forms show activity towards *p*-coumaric, ferulic isoferulic, caffeic sinapic and 3,4 dimethoxycinnamic acids and do not show major differences in their substrate specificities although the lower MW form has a relatively higher activity towards sinapic and 3,4-dimethoxycinnamic acids.

DISCUSSION

The two isoenzymes here reported from peas appear similar to the two forms I and II reported by Knobloch and Hahlbrock [8] using soyabean extracts. Peak I in the present paper corresponds to soyabean 'ligase I' which is active towards sinapic acid and methoxylated cinnamic acids while peak II corresponds to soyabean 'ligase II' which is inactive with sinapic acid. However the behaviour of the two isoenzymes from pea on DEAE cellulose is rather different to that of the soyabean enzyme in that the two forms elute in the salt gradient on DEAE cellulose in the reverse order. Ranjeva *et al.* [9] using leaves of *Petunia* have separated two fractions of hydroxycinnamate : CoA ligase eluting from DEAE-cellulose at

0.25 M and 0.65 M KCl. The form eluting at lower salt concentration is similar to the isoenzyme I from pea seedlings in having high activity with sinapic acid. It is interesting that this fraction also has high activity towards caffeic acid and in more recent work [12] this form eluting from DEAE at low salt concentrations has been resolved into a caffeic acid specific enzyme thought to be involved in chlorogenic acid biosynthesis and a sinapic acid specific form thought to be involved in lignification.

The pH optimum of 7.5 for peak II obtained after chromatography of pea extracts on DEAE cellulose is in accordance with the values of 7.5–7.8 obtained for Petunia [9] and the soyabean 'ligase II' [8]. Peak I which also has an optimum at pH 7.5 differs slightly from that of Petunia (7.5–7.8) but more so from the 'ligase I' of soyabean (8.3).

The pattern of elution of the pea ligase from Ultragel AcA 34 suggests that the enzyme exists in a number of MW forms which have activity towards both *p*-coumaric acid and sinapic acid and do not differ significantly in overall substrate specificity. The elution position of the high MW fraction indicates a MW of 75 000 ± 5 000 and this was confirmed by elution from calibrated columns of Sephadex G100. The elution positions of the other two slowly eluting fractions from Ultragel suggests that the MW fraction indicates a MW of 75 000 ± 5000 and cellulose suggest that there are two distinct isoenzymes of hydroxycinnamate CoA ligase in pea seedlings which differ in specificity with respect to certain methoxylated substrates and which each exist in a number of MW forms which are interconvertible. The factors involved in the association–dissociation reactions leading to the interconversion of the high and low MW forms are not understood but experiments suggest that freezing the enzyme promotes aggregation of smaller units into the high MW form. The two forms separated on DEAE-cellulose are not interconvertible as judged by rechromatography on DEAE-cellulose, indicating that there are two isoenzymes of hydroxycinnamate CoA ligase in the pea seedling which differ in ionic properties and in their substrate specificity.

EXPERIMENTAL

In the survey of the substrate specificity of the hydroxycinnamate CoA ligase from various plant sources the enzyme fraction used was prepared using $(NH_4)_2SO_4$ ppn, desalting on Sephadex G-25 and bulk purification on DEAE-cellulose (DE-23) by the method previously used for swede disk tissue [10].

Extraction and purification of hydroxycinnamate CoA ligase from pea shoots. 200 g etiolated pea shoots were homogenised in an Ato-mix homogeniser in 600 ml of a medium containing 0.1 M Tris pH 7.45, 0.25 M Sucrose, 1mM EDTA, 1mM DTE and 1% Polyclar AT. The homogenate was filtered through miracloth and $(NH_4)_2SO_4$ added to give 50% satn. After centrifugation at 40 000 *g* for 45 min. the supernatant was decanted and further $(NH_4)_2SO_4$ added to give 75% satn. The ppd protein was collected by centrifugation at 40 000 *g* for 30 min. The ppt. was resuspended in the minimum vol. of 0.005 M Tris pH 7.45–1mM DTE and desalted on a column of Sephadex G-25 (medium grade 2.5 × 35 cm) previously equilibrated with the same buffer. The protein fraction was collected and applied to a column of DEAE-cellulose (Whatman DE52, 1.5 × 12 cm) equilibrated with 0.005 M Tris pH 7.45–1 mM DTE. The column was washed with the same buffer and then eluted with a linear salt gradient between 0–0.5 M KCl in 0.005 M Tris pH 7.45–1mM DTE in a total vol. of 200 ml. 3 ml fractions were collected and assayed for hydroxycinnamate : CoA ligase. All stages in the enzyme purification were carried out at 0–2°. For separation of the various MW forms of the ligase, the 50–75% $(NH_4)_2SO_4$ fraction after clarification by centrifugation at 40 000 *g* for 30 min was applied in a vol. of 5 ml to a column of Ultragel AcA 34 (LKB Ltd., 2.5 × 35 cm) equilibrated with 0.2 M Tris pH 7.45–1mM DTE. The column was eluted with this same buffer at a rate of 23 ml/hr. 3 ml fractions were collected and assayed for ligase activity. The Ultragel column was calibrated for MW by determining the elution vols of proteins of known MW. The standard proteins used included carboxypeptidease A, hexokinase, glucose 6-phosphate dehydroganse, lactate dehydrogenase, alcohol dehydrogenase, fumarase and pyruvate kinase. The elution pattern of these enzymes was determined using the assay methods of ref. [13]. In similar experiments, the MW's of the ligase fraction was confirmed by applying the $(NH_4)_2SO_4$ fraction to a calibrated column of Sephadex G100 (superfine 2.5 × 35 cm). For chromatogrpahy on hydroxyapatite (Bio-gel HT) the 50–75% $(NH_4)_2SO_4$ fraction was desalted on Sephadex G25 and equilibrated in 0.01 M KH_2PO_4 pH 6.8. This fraction was applied to the hydroxyapatite column (1.5 × 15 cm) and eluted with a linear gradient of phosphate buffer pH 6.8 between 0.01–0.2 M. Similarly for chromatography on CM-cellulose (Whatman CM-52), the $(NH_4)_2SO_4$ fraction was desalted on Sephadex G25 and equilibrated with 0.005 M phosphate pH 6.5. This fraction was applied to the CM cellulose column (1.5 × 12 cm) and the column eluted with the same buffer.

Assay method for hydroxycinnamate : CoA ligase. The direct spectrophotometric method was used for the assay of hydroxycinnamate CoA ligase as previously described [12] but in addition 1 mg/ml BSA was included in the assay mixture. When substrates other than *p*-coumaric acid were used, the wavelengths determined by Gross and Zenk [14] for the maximum of the difference spectrum between cinnamic acids and their CoA esters were used. The extinction coefficients determined in ref. [14] were used to calculate rates of formation of the CoA esters.

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