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THE *p*-COUMARYL CoA LIGASE OF POTATO TUBERS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato tuber; *p*-coumaryl CoA ligase; substrate specificity.

Abstract—The demonstration of activity of *p*-coumaryl CoA ligase in extracts of aged potato disks proved difficult owing to the presence of extremely high levels of apyrase which caused rapid hydrolysis of ATP, a co-factor for ligase activity. This problem was largely overcome by including an inhibitor of apyrase, sodium fluoride in the ligase assay and by initiation of the reaction with ATP. A method for the separation of apyrase and *p*-coumaryl CoA ligase by chromatography on DEAE-cellulose is described. *p*-Coumaryl CoA ligase was not detectable in freshly prepared disks of potato tubers. However on ageing in the light a large increase in the activity of this enzyme occurs. The enzyme of aged potato disks shows high activity with *p*-coumaric, ferulic, caffeic and with *m*- and *p*-methoxycinnamic acids. However the affinity of the enzyme for the methoxy derivatives is much lower than for cinnamic acids bearing free hydroxyl groups.

INTRODUCTION

Enzymes catalysing the activation of cinnamic acid derivatives to form their CoA thioesters have been found in a number of plant tissues [1–9]. All these enzymes are specific for CoA and ATP and are activated by Mg^{2+} but differ somewhat in their substrate specificity. The enzymes from swede root [6] and *Forsythia* [1] are specific for cinnamic acids with free phenol groups, while the enzyme from other sources will also activate cinnamic acid itself and methoxycinnamic acids. Recent studies [10] suggesting the resolution of cinnamyl CoA ligase into two isoenzymes showing different substrate specificities indicate that there may be two ligase enzymes, one specific for hydroxycinnamic acids and the other activating both phenolic and non-phenolic cinnamic acid derivatives.

Stöckigt and Zenk [11] have indicated the possible involvement of the CoA thioester of caffeic acid as an intermediate in the biosynthesis of chlorogenic acid in *Nicotiana* tissue cultures. This finding has led us to investigate the activity of the cinnamyl CoA ligase in potato tuber disks under conditions in which chlorogenic acid biosynthesis is known to be accelerated [12]. This has proved to be difficult owing to the presence of high ATPase activity in the extracts of this tissue. A partial solution to the problem is described and the properties of the enzyme from potato are considered in relation to its possible involvement in chlorogenic acid biosynthesis and in relation to its substrate specificity.

RESULTS

Extracts were made from disks of potato tubers which had been incubated in the light under conditions in which chlorogenic acid biosynthesis is activated [12]. When the extracts fractionated by $(NH_4)_2SO_4$ were tested in the cinnamyl CoA ligase assay [6], a small activity was found in the 0–35% fraction but none in the 35–75% fraction. When the two fractions were separately desalted on Sephadex G25 and chromatographed on DEAE-cellulose columns using a linear salt gradient, each $(NH_4)_2SO_4$ fraction gave a peak of ligase activity which eluted from the DEAE-cellulose column in the same position on the gradient (and this position closely corresponds to the

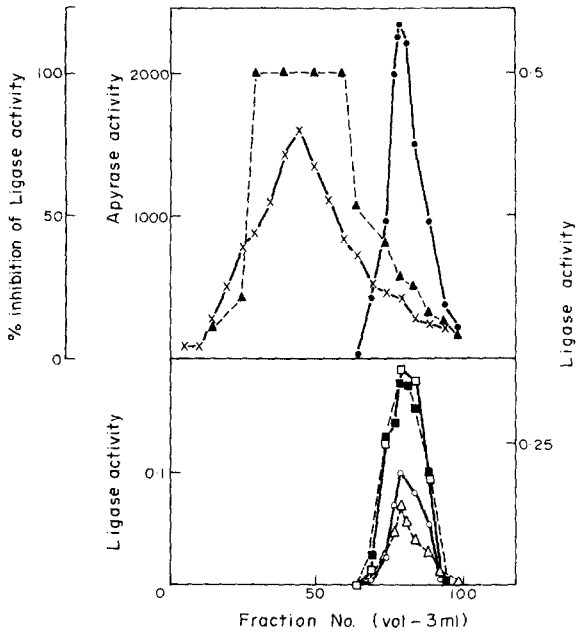

Fig. 1. The separation of enzyme activities from extracts of potato tuber disks aged in light: × apyrase; ▲ inhibition of purified swede *p*-coumaryl ligase by fractions of potato extracts of the DEAE-cellulose chromatography (% inhibition); ● *p*-coumaryl CoA ligase; ⊡ ferulyl CoA ligase; ■ caffeyl CoA ligase; △ *p*-methoxycinnamyl CoA ligase; ⊙ *m*-methoxycinnamyl CoA ligase. Enzyme activity for apyrase is expressed as μgP, released/10 min/fraction under standard assay conditions. For ligase activities the enzyme unit is defined as the amount of enzyme causing a change of absorbance of 1 per min under standard conditions.

ligase activity of swede root tissue, see [7]). However, the total activity in the 35–75% $(NH_4)_2SO_4$ fraction far exceeded that in the 0–35% fraction. These results indicated that the 35–75% fraction contains an inhibitor of the assay which separated from the ligase peak on the DEAE-cellulose column. Figure 1 shows that when the 35–75% $(NH_4)_2SO_4$ fraction was subjected to gradient elution from DEAE-cellulose and the fractions of the eluate were tested for their ability to inhibit purified swede ligase, there was a peak of inhibitory activity which preceded the elution position of the potato ligase. The inhibitory activity was shown to be due to a non-dialysable heat labile factor and could be partially reversed by increasing the ATP concentration in the assay. Subsequently (Fig. 1) it was shown that a peak of ATPase activity closely corresponded to the peak of inhibitory activity. This indicated that the inhibitor is in fact, ATPase in spite of the fact that the standard assay contains an excess of ATP (2·5 μmol/ml) in relation to the K_m of the ligase for ATP of 0·19 mM [6].

The properties of the ATPase were studied and the peak after DEAE-cellulose chromatography was used for this purpose. The kinetics of hydrolysis of ATP were followed and as the yield of Pi exceeded 1 mol/mol ATP it was assumed that the activity involved an apyrase of the type described by Molnar and Lorand [13] and Traverso-Cori *et al.* [14]. The apyrase was activated by Mg^{2+} and inhibited by EDTA and the EDTA inhibition was reversible by Co^{2+}, Ni^{2+} and Mn^{2+} as well as by Mg^{2+}. It had previously been shown that Co^{2+}, Ni^{2+} and Mn^{2+} would replace Mg^{2+} in the ligase assay [6]. Table 1 shows that the apyrase was inhibited by 60% by NaF at 40 mM with no effect on ligase activity. In sub-

Table 1. Properties of apyrase of potato tuber tissue and the effect of sodium fluoride on apyrase and cinnamyl CoA ligase activity

	Apyrase activity (nkat/mg protein)		
Complete assay	4·5		
$-Mg^{2+}$	3·0		
$-Mg^{2+}$ + EDTA	0·5		
$+Mg^{2+}$ + EDTA	6·2		
Concentration NaF (mM)		**Inhibition (%)**	**Relative *p*-coumaryl CoA ligase activity**
0	4·8	—	100
10	2·7	44	—
20	2·5	46	—
40	2·0	56	102
100	1·5	68	92

Table 2. The substrate specificity of *p*-coumaryl CoA ligase of potato tuber disks

Substrate*	Enzyme activity in standard assay ($s = 0{\cdot}5$ mM) pkat/mg protein	V_m	K_m (μM)
Cinnamic acid	0	—	—
p-Coumaric acid	36·2	38·6	6·2
o-Coumaric acid	7·3	—	—
m-Coumaric acid	26·0	—	—
p-Methoxycinnamic acid	5·7	12·9	690
m-Methoxycinnamic acid	7·5	12·3	336
Caffeic acid	18·5	24·5	16·1
Ferulic acid	28·0	33·6	13·7
Isoferulic acid	27·5	—	—
2,4-Dimethoxycinnamic acid	0·8	—	—

* There was no activity towards *o*-methoxycinnamic, 3,4-dimethoxycinnamic, 3,4,5-trimethoxycinnamic or sinapic acids.

sequent experiments, NaF at 40 mM was included in all ligase assays on fractions prior to resolution of the apyrase on DEAE-cellulose. The ligase assay [6] was also modified by initiating the reaction with ATP rather than CoA to minimise contact of the ATP with the apyrase.

Table 2 shows the activity of potato ligase enzyme with different cinnamic acid substrates. As with the enzyme from swede roots, the potato ligase shows highest activity with *p*-coumaric acid and has the highest affinity for this substrate. Ferulic and caffeic acids are both activated at high rates and the enzyme has a high affinity for these substrates. Isoferulic and *m*-coumaric acids are also activated at relatively high rates. However, the potato enzyme differs from the swede enzyme in showing activity towards *p*-methoxycinnamic, *m*-methoxycinnamic acids and to a lesser extent towards 2,4-dimethoxycinnamic acid. No activity towards cinnamic acid itself, towards the other di- or tri-methoxy derivatives tested or towards sinapic acid was observed. The V_m of activity towards the methoxycinnamic acids is roughly a 1/3 of that towards *p*-coumaric acid. However, the enzyme has between 50–100 times greater affinity for *p*-coumaric acid than either *p*- or *m*-methoxycinnamic acids.

Figure 1 shows that the elution of the activities towards *p*-coumaric, ferulic and caffeic acids and towards *m*- and *p*-methoxycinnamic acids occurs in the same position of the salt gradient. In chromatographic studies of the potato ligase, there was no evidence for the presence of isozymes.

The *p*-coumarate ligase activity is not detectable in freshly prepared disks but there is a large increase in activity (18·4 pKat/mg protein at 24 hr) during ageing in the light. Extracts of initial disks were subjected to the same procedure including chromatography on DEAE-cellulose as were extracts of aged disks, but with initial disks no ligase activity was detected. There is also induction of phenylalanine ammonia lyase (PAL) activity (from 2·6 to 51·1 pKat/mg protein during 24 hr), confirming the findings of Zucker [12].

DISCUSSION

The present paper shows that during the ageing of disks of potato tuber tissue under conditions in which chlorogenic acid biosynthesis is activated and when there is an increase in PAL activity, there is also an increase in the activity of a *p*-coumaryl CoA ligase. The difficulty of demonstrating ligase activity in crude potato extracts was due to the presence of high levels of apyrase activity. The inhibition results from a combination of the hydrolysis of the co-factor, ATP and the formation of ADP and AMP which inhibit ligase activity [6]. A partial solution to the problem has been found by including NaF, an inhibitor of apyrase, in the ligase assays and by initiating the reaction with ATP rather than by CoA. An almost complete resolution of apyrase and the cinnamyl CoA ligase was obtained after chromatography on DEAE-cellulose.

Four enzymes involved in the biosynthesis of phenylpropanoids have been previously shown to increase during the ageing of disks of potato tuber tissue. These are shikimate dehydrogenase, PAL, cinnamic acid 4-hydroxylase and *O*-methyltransferase [12,15,16]. The increase in PAL has been confirmed in this paper and in addition a large increase in *p*-coumaryl CoA ligase has been demonstrated. This enzyme has a higher affinity for *p*-coumaric acid than for caffeic acid and this could suggest the involvement of *p*-coumaryl CoA as an intermediate in *p*-coumarylquinic acid biosynthesis and its subsequent hydroxylation to chlorogenic acid [17].

The substrate specificity of the potato ligase differs from that of the enzyme of swede roots in showing marked activity towards *m*-and *p*-methoxycinnamic acids. The activity of the potato

enzyme towards *m*-methoxycinnamic acid is roughly one third that towards its most active substrate, *p*-coumarate. However, the affinity for the methoxycinnamic acids is very low compared with that towards *p*-coumaric acid. The evidence from the DEAE-cellulose chromatography suggests that a single enzyme of relatively wide specificity is involved in the activation of cinnamic acids. Unpublished kinetic data using purified swede enzyme also suggests that a single enzyme site is responsible for the activity with the various cinnamic acid substrates.

EXPERIMENTAL

Disks (2 × 10 mm) of potato tubers (var Redskin) were incubated in moist chambers illuminated with fluorescent light for 24 hr at 20°. Samples of 80 g disks were homogenised in a medium containing 0·25 M sucrose, 0·10 M Tris–HCl, 0·001 M EDTA, 1 mM dithiothreitol, 2 mM $Na_2S_2O_5$, Polyclar AT 1%, at pH 7·8. Procedure for the purification of the ligase enzyme was essentially as described for swede ligase [7] with the exception that 2 mM $Na_2S_2O_5$ was included in the elution buffers while the $(NH_4)_2SO_4$ fractions used were 0–35% and 35–75% saturation rather than 0–55 and 55–75%. Apyrase activity was measured by the release of Pi during a 10 min incubation at 30° of a mixture containing 2·5 μmol Mg^{2+}, 2·5 μmol ATP, and 0·2 M Tris pH 7·45 and enzyme to a final vol of 1 ml. The incubation was stopped by addition of 10% TCA and the Pi released measured by the method of Atkins [18]. Assay for ligase activity was essentially that used earlier [6] except that the reaction was initiated with ATP rather than CoA and NaF at a concentration of 40 mM was included in assays prior to DEAE-cellulose fractionation. The protein content of the various fractions was measured [19] after precipitating with 20% TCA and dissolving the resulting precipitate in 0·1 N NaOH. PAL activity was measured as previously described [20].

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