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PHOTOCONTROL OF CHLOROGENIC ACID BIOSYNTHESIS IN POTATO TUBER DISCS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; biosynthesis; polyphenols; photocontrol; chlorogenic acid; phenylalanine ammonia-lyase; cinnamic acid 4-hydroxylase.

Abstract—The appearance of phenylalanine ammonia-lyase activity and the accumulation of chlorogenic acid in potato tuber discs are stimulated by illumination with white light, whereas the appearance of cinnamic acid 4-hydroxylase activity is unaffected by illumination. The photosensitive step in chlorogenic acid biosynthesis may be by-passed by treatment of discs with exogenous supplies of cinnamic acid, whereas treatment of discs with phenylalanine does not isolate the photosensitive step. Therefore, the site of photocontrol of chlorogenic acid biosynthesis in potato tuber discs is the reaction catalysed by phenylalanine ammonia-lyase. Cinnamic acid 4-hydroxylase activity *in vitro* is unaffected by *p*-coumaric acid, caffeic acid or chlorogenic acid. Phenylalanine ammonia-lyase activity *in vitro* is sensitive to inhibition by cinnamic acid. The *in vitro* properties of the two enzymes are also consistent with the hypothesis that phenylalanine ammonia-lyase rather than cinnamic acid 4-hydroxylase is important in the regulation of chlorogenic acid biosynthesis in potato tuber discs.

INTRODUCTION

Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) and cinnamic acid 4-hydroxylase (CA4H, E.C. 1.14.13.11) catalyse the first two reactions in the biosynthesis from phenylalanine of a wide variety of phenylpropanoid compounds including lignin, esters of caffeic acid and flavonoids [1, 2]. The classic work of Zucker established that PAL activity appears in potato (*Solanum tuberosum*) tuber tissue following disc excision and that the appearance of activity is stimulated by illumination [3]. The accumulation of chlorogenic acid (CGA), the major low MW biosynthetic product of phenylalanine in potato, is also stimulated by illumination [4]. The magnitude of the increase in CGA content is closely related to the maximum level of PAL activity attained in discs illuminated for varying periods [3]; it was suggested that the level of PAL controlled the flux through the pathway from phenylalanine to CGA during disc ageing, and that PAL was therefore the primary site for the photocontrol of CGA accumulation [3]. Current evidence suggests that a likely major route to CGA involves, in addition to PAL and CA4H, a "*p*-coumarate hydroxylase", a caffeoyl: CoA ligase (see refs. [1, 2]) and a caffeoyl quinate: CoA transferase [5]. Recent experiments using a variety of biological systems that synthesise phenylpropanoids have shown that PAL and some succeeding enzymes may change their levels of activity coordinately. For example, in illuminated parsley cell suspension cultures, CA4H or *p*-coumaroyl: CoA ligase, rather than PAL, could be the primary control point in phenylpropanoid biosynthesis since all three enzymes exhibit parallel changes in their activities [6]. These arguments might equally well be applied to the control of CGA biosynthesis in potato. This paper re-examines the regulation of the accumulation of CGA in potato tuber discs, with special reference to the roles of PAL and CA4H and the site of photocontrol.

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Abbreviations used: PAL: Phenylalanine ammonia-lyase; CA4H: cinnamic acid 4-hydroxylase; CGA: chlorogenic acid.

RESULTS

Correlation of PAL and CA4H activity with CGA accumulation in potato tuber discs

There is no spectrophotometrically detectable PAL activity in dormant King Edward potato tuber discs, but a low CA4H activity is present. The appearance of CA4H activity in excised discs (2 mm thick) is insensitive to illumination for up to at least 47 hr, whereas the appearance of PAL activity is greatly stimulated by illumination (×3.5 after 14 hr incubation) and is consistently higher throughout the time-course in illuminated discs than in comparable discs incubated in darkness (Fig. 1). Despite this differential effect of illumination on the appearance of PAL and CA4H activities, the respective time-courses suggest some degree of coordination between the levels of activity of the two enzymes (Fig. 1). Thus in both illuminated and non-illuminated discs, PAL and CA4H activities increase after a lag of about 5 hr to reach maximum levels after 14 hr and subsequently decline to significantly lower levels.

Parallel studies on the time-course of accumulation of CGA in potato tuber discs were undertaken to allow comparison of the rate of CGA accumulation with the appearance of PAL and CA4H activities (Fig. 1). Significant amounts of CGA are found in freshly prepared discs, a typical value being 110 nmol/g fr. wt. In non-illuminated discs, a slow linear increase in CGA content is observed for 43 hr following disc excision. In illuminated discs there is a rapid accumulation of CGA

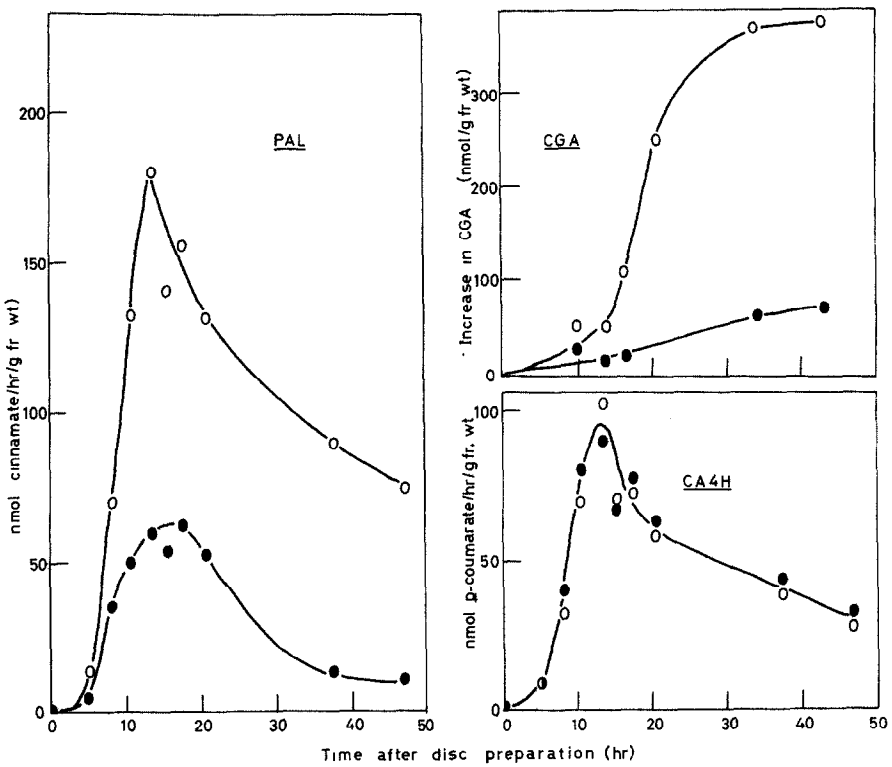

Fig. 1. Time-course of the appearance of PAL and CA4H activities and of the accumulation of CGA in illuminated (O) and non-illuminated (●) potato tuber discs.

between 10 and 35 hr after disc excision, the maximum rate of accumulation occurring after 18 hr. Following this phase of rapid accumulation, CGA levels continue to rise at a slow rate, similar to that observed with comparable non-illuminated discs. The temporal relationship between the appearance of PAL and CA4H activities in illuminated discs and the accumulation of CGA (Fig. 1) is that expected for the accumulation of a product in response to the development of the biosynthetic capability without a similar increase in catabolic capability.

The maximum rate of accumulation of CGA in illuminated potato tuber discs is approximately 38 nmol/hr/g fr. wt and this can be compared with the maximum catalytic activity of PAL and CA4H extracted from illuminated potato tuber discs of 135 nmol/hr/g fr. wt and 75 nmol/hr/g fr. wt respectively (assuming a Q_{10} of 2.1–2.2 for both reactions in order to convert rate at assay temperature to rate at disc incubation temperature [7, 8]). Thus the extractable levels of activity of PAL and CA4H appear sufficient to account for the observed rate of accumulation of CGA. Furthermore, assuming that the enzymes do not operate at V_{max} *in vivo* and may also be subject to inhibition, it is likely that CGA represents the major product associated with PAL and CA4H activity.

Effect of exogenous supplies of L-*phenylalanine and trans-cinnamic acid on the accumulation of CGA*

In the presence of 30 mM phenylalanine in the incubation medium, the level of CGA present after 24 hr in both illuminated and non-illuminated discs was increased compared to "light" and "dark" controls incubated on water alone (Table 1). Phenylalanine plus light had a greater effect than either phenylalanine or light alone. With exogenously supplied cinnamic acid, the accumulation of CGA was also stimulated in both illuminated and non-illuminated discs, but contrasting strongly with the phenylalanine data, cinnamic acid plus light had the same effect as cinnamic acid alone (Table 1). Thus the ability of light to increase CGA levels over control values is abolished by external cinnamic acid, but not by external phenylalanine.

Changes in the level of cinnamic acid when air is removed from discs and subsequently re-introduced

On removal of air (O_2 is a substrate for CA4H, [8]), the rate of accumulation of cinnamic acid in discs supplied with exogenous phenylalanine is fast at first, (12 nmol/hr/g fr. wt), but subsequently declines as the level of cinnamic acid approaches a plateau. On releasing the constraint by re-introducing air, the level of cinnamic acid rapidly declines (9 nmol/hr/g fr. wt), implying that the catalytic activity of CA4H *in vivo* is sufficiently rapid

Table 1. Effect of exogenous supplies of phenylalanine and cinnamic acid on the accumulation of CGA in potato tuber discs aged for 24 hr

	CGA Accumulation (nmol/g fr wt)		Stimulatory effect of illumination
	Illuminated	Non-illuminated	
Experiment 1			
Water	180	30	150
Cinnamic acid (2 mM, pH 7)	345	330	15
Experiment 2			
Water	270	30	240
Phenylalanine (30 mM)	375	210	165

to hydroxylate the cinnamic acid produced by PAL activity, even under conditions where PAL may be operating near V_{max}

In vitro *properties of PAL and CA4H*

Kinetic analysis of PAL activity in extensively dialysed extracts of incubated potato tuber discs was undertaken at pH 8.8, which is the pH optimum of the enzyme [9], and at pH 7. At pH 8.8, PAL does not exhibit Michaelis-Menten kinetics with respect to phenylalanine. The apparent K_m at low concentrations of phenylalanine is about an order of magnitude smaller than the apparent K_m at higher substrate concentrations. Estimates of K_m (high substrate) and K_m (low substrate) are 0.88 and 0.03 mM respectively, although there is a considerable inherent error in the estimation of limiting slopes from curves of this kind. This unusual kinetic profile ("negative rate cooperativity") is identical with extracts made from discs incubated for 15 hr under illumination, 40 hr under illumination and 15 hr in darkness. The inhibition of PAL at pH 8.8 by the product cinnamic acid is of the mixed type. The slope of the Dixon plot (v_0^{-1} vs inhibitor concentration at various values of substrate concentration) increases with the concentration of cinnamic acid, this curvature being more pronounced the lower the concentration of phenylalanine (Fig. 2).

At pH 7, the enzyme exhibits Michaelis–Menten kinetics with respect to phenylalanine (K_m = 0.53 mM). The inhibition of the enzyme by cinnamic acid is competitive with respect to phenylalanine (K_i = 55 μM). *p*-Coumaric acid, caffeic acid, quinic acid and CGA have no effect on PAL activity at concentrations where cinnamic acid exerted strong inhibition (Table 2). However, *o*-coumaric acid was an inhibitor of PAL activity although it was not as effective as cinnamic acid at equivalent concentrations (Table 2).

CA4H activity is localised almost exclusively in the microsomal fraction of extracts from aged potato tuber discs ([10] and C. J. Lamb and P. R. Rich unpublished results). The enzyme follows Michaelis–Menten kinetics with respect to cinnamic acid at saturating concentrations of NADPH, giving a K_m of 11 μM. Furthermore, CA4H activity in crude extracts or microsomal preparations of aged potato tuber discs is not inhibited by *p*-coumaric acid, caffeic acid or CGA at concentrations up to at least 66 μM, either in the presence of 0.66 mM or 33 μM cinnamic acid.

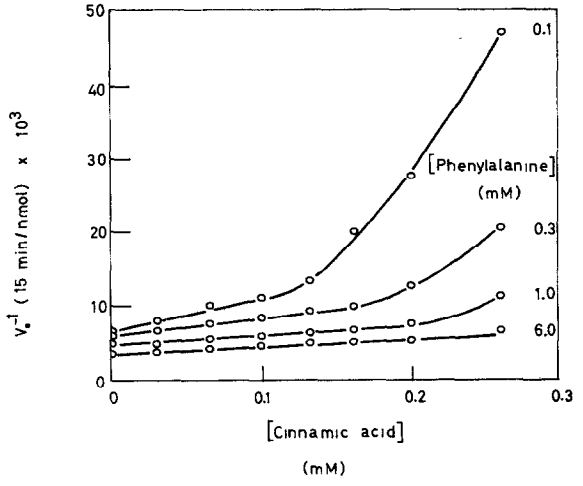

Fig. 2. Dixon plot of the initial velocity of cinnamic acid formation as a function of cinnamic acid concentration in the presence of various concentrations of phenylalanine. PAL was extracted from potato tuber discs that had been aged for 15 hr under constant illumination, into 0.1 M borate buffer (pH 8.8, Na as the counter ion) containing 2-mercaptoethanol (1 mM). Extracts (25 ml samples) were dialysed against 2 l. of the same buffer for 8 hr and then transferred to a further 2 l. of buffer for 16 hr before kinetic analysis.

Table 2. Effect of various compounds on PAL activity *in vitro*

Compound	Concentration of compound (μM)	Phenylalanine concentration (mM)	PAL activity (% control)
Cinnamic acid	33	0.1	88
	66	0.1	72
	100	0.1	63
	133	0.1	36
	200	0.1	25
p-Coumaric acid	33	0.1	102
	66	0.1	102
	133	0.1	100
	33	10	101
	66	10	96
o-Coumaric acid	33	0.1	92
	66	0.1	84
	100	0.1	76
	133	0.1	67
	200	0.1	38
Caffeic acid	33	0.1	104
	66	0.1	104
	133	0.1	99
Quinic acid	66	0.1	97
Chlorogenic acid	33	0.1	101
	66	0.1	98

PAL was extracted from potato tuber discs that had been aged for 15 hr under constant illumination. Extracts were extensively dialysed against 0.1 M borate buffer pH 8.8 containing 2-mercaptoethanol (1 mM) and PAL activity was estimated in the presence of various compounds. The results are expressed as a percentage of the control.

DISCUSSION

On the basis of the time-course of appearance of PAL and CA4H activities and the accumulation of CGA in potato tuber discs, either PAL or CA4H could be considered as the site of primary control of flux through the pathway leading to CGA biosynthesis. However, observation of the stimulatory effect of continuous illumination on the appearance of PAL activity and accumulation of CGA, and the lack of effect of illumination on the appearance of CA4H activity allows the conclusion that CA4H is not the site of photocontrol of CGA biosynthesis. Furthermore, exogenous supplies of cinnamic acid appear to isolate the step in the biosynthesis of CGA that is photosensitive (Table 1). In contrast, photocontrol is still present when phenylalanine is supplied exogenously. This is inconsistent with the idea that some stage in the biosynthesis of CGA later than the deamination of phenylalanine to cinnamic acid is the step of CGA accumulation that is subject to photocontrol. Similarly, the incorporation of phenylalanine-^{14}C but not cinnamic acid-^{14}C into the flavonoids of terminal pea buds was subject to photocontrol [11]. It is concluded that either the supply of phenylalanine, or more probably, the level of PAL activity is the site for photocontrol of CGA accumulation in potato tuber discs. The latter alternative is favoured because of the close relationship between the appearance of PAL activity and the time-course of CGA accumulation. The rate of accumulation of cinnamic acid in

potato tuber discs in response to anaerobiosis and the rate of disappearance of cinnamic acid on re-introduction of air provides an estimate of the relative activities of PAL and CA4H *in vivo*. These results support the hypothesis that CA4H is not rate-limiting in CGA biosynthesis in potato tubers, similar results and conclusions have been reported using strawberry leaf discs [12].

The *in vitro* properties of PAL are consistent with the enzyme having a regulatory function. The product, cinnamic acid, is inhibitory at all concentrations of phenylalanine (substrate) examined, and significant inhibition is observed at concentrations of cinnamic acid that would feasibly occur *in vivo*. Furthermore, the shape of Dixon plots for cinnamic acid inhibition of PAL activity implies that PAL activity is very sensitive to changes in the concentration of cinnamic acid. Negative rate cooperativity of PAL activity with respect to phenylalanine is observed with purified PAL from potato [9], wheat [13], pea [14] and gherkin [15]. We have shown that negative rate cooperativity is associated with sensitive product inhibition [16] and therefore, the unusual kinetics exhibited by PAL may relate to the control of phenylpropanoid biosynthesis by product inhibition of PAL [16]. The lack of inhibition of CA4H activity by *p*-coumaric acid, caffeic acid and CGA suggests that modulation of CA4H activity *in vivo* by these compounds is not a component of the fine control of CGA biosynthesis in potato tubers. These observations lend further support to the idea that modulation of CA4H is not of primary importance in the control of CGA biosynthesis in potato.

On the basis of the different effects of illumination, Camm and Towers [17] concluded that the mechanisms of control of PAL and CA4H activities in potato tuber discs must be different from the mechanisms operating in parsley [6] and buckwheat [18]. However, the kinetics of the appearance and disappearance of PAL and CA4H activities in potato tuber discs are sufficiently similar to conclude that some form of integrated control mechanism operates, as in other systems, although there is no demanding evidence to indicate at which biochemical level integration may occur. Clearly however, any model for such a control system must allow for the differential effect of illumination in potato tuber discs.

EXPERIMENTAL

Potato tubers (*Solanum tuberosum* cv. King Edward) were obtained from the local market and stored at 15°, in darkness. Expts were always performed on tubers that had been stored for less than 1 month. Tubers weighing between 0.1 and 0.2 kg were used as the source of material for all experiments. Columns of pith tissue 20 mm in diam were obtained using a cork-borer and slices 2 mm thick were cut using a hand microtome. Discs were washed in H_2O before placing in petri dishes (4 discs/dish), moistened with 5 ml H_2O or specified soln and incubated at 25° in darkness or under white light (5400 lx at the disc surface).

Extraction of enzymes. 4 discs were used for each assay. Before extraction, discs were washed ×3 with H_2O, blotted on filter-paper and weighed. Extracts were prepared by grinding discs in a chilled pestle and mortar with 5 ml cold 0.05 M Pi buffer (pH 7.6, Na as the counter ion), containing 2-mercaptoethanol (1 mM). A small number of Ballotini beads (No. 12) were included to aid extraction. Cell debris was removed by centrifugation at 500 *g* for 5 min and the resultant supernatant used for assays of both PAL and CA4H. Microsomes were isolated and stored as described in ref. [19].

Enzyme assays. (i) CA4H activity was assayed at 30° by a stopped spectrophotometric assay [20]. (ii) In expts comparing PAL and CA4H activities in total extracts, PAL activity was assayed at 30° by the method of ref (21). The pH for assay was adjusted by addition of 1 ml of 0.3 M borate buffer (pH 8.95, Na as the counter ion) to 1 ml of total extract (0.05 M NaPi buffer, pH 7.6). For the kinetic analysis of PAL, extracts (25 ml) were dialysed against 2 l. of 0.1 M borate buffer (pH 8.8, Na as the counter ion), containing 2-mercaptoethanol (1 mM) for 8 hr and then transferred to a further 2 l. of buffer for 16 hr. Buffer solns were stirred continuously and the dialysis was performed at 4°. PAL activity of the dialysed extracts was assayed using a continuous spectrophotometric assay at 290 nm, the temp. of the incubation being 40° [22].

Anaerobiosis. Removal of air from discs was achieved by evacuation as described in ref. [12].

Cinnamic acid estimation. Cinnamic acid was extracted from discs with 50% aq MeOH (5 discs/10 ml) and cell-debris removed by centrifugation. The supernatant was acidified, any resultant ppt. removed by centrifugation, and the resultant supernatant extracted ×3 with 1 vol. EtOAc. The EtOAc extracts were pooled, evaporated to dryness under vacuum and re-dissolved in EtOH. Cinnamic acid was resolved by PC using C_6H_6–HOAc–H_2O (2:2:1) as the mobile phase [8]. Cinnamic acid was eluted with EtOH and estimated spectrophotometrically at 270 nm against appropriate blanks.

CGA estimation. CGA was extracted and estimated by the procedure of ref. [23].

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