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AN INACTIVATOR OF PHENYLALANINE AMMONIA-LYASE FROM GHERKIN HYPOCOTYLS

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; gherkin; PAL; enzyme inactivation.

Abstract—A factor capable of the reversible inactivation of PAL *in vitro* has been demonstrated in extracts of gherkin hypocotyls. Kinetics of the interaction between enzyme and inactivator indicate that PAL and the factor form a freely reversible complex. The properties of the inactivator are discussed in relation to its proposed role in the regulation of PAL activity in dark- and light-grown tissue.

INTRODUCTION

Recently there has been increasing interest in the possible role of specific inhibitors and inactivators as the regulators of enzyme activity in plant cells in response to external stimuli [1, 2]. The number of known reversible enzyme inactivators is small [3–5] but there is considerable evidence for the *in vivo* operation of activation/inactivation in several plants [1, 2]. In gherkin seedlings, for example, the activity of phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5) is greatly increased by treatment with the protein synthesis inhibitor cycloheximide [6] suggesting that continued protein synthesis may be necessary to maintain the enzyme in an inactive state. Evidence for the existence of an inactive form of PAL in extracts of radish seedlings has been published [7]. Recently, Attridge and Smith [8] have shown by density labelling that both light-mediated and cycloheximide-initiated increases in PAL activity in gherkin seedlings are due to activation of pre-existing enzyme. In this paper we report the extraction of a factor from gherkin hypocotyls which reversibly inactivates PAL *in vitro*.

RESULTS

Figure 1 shows the effect of pre-incubation of the standard enzyme extract with the supernatant of an extract prepared from seedlings irradiated with blue light for 12 hr. Clearly the activity of the PAL preparation declines with increasing period of incubation with the inactivator. Maximum ability to inactivate PAL was observed in preparations taken from hypocotyls 12 hours after the onset of continuous blue irradiation; by this time the *in vivo* PAL levels in the hypocotyl had declined to the initial dark level [6].

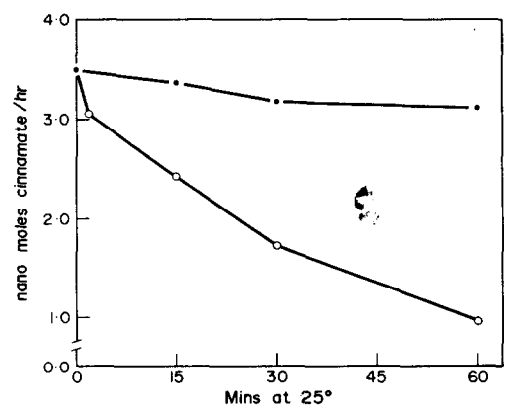

Fig. 1. Effect of inactivator on PAL activity *in vitro*. Inactivator was extracted from seedlings treated with 12 hr blue light. The standard PAL extract was obtained from material given 4 hr blue light. Open circles, PAL + inactivator; solid circles, PAL + buffer (control). PAL activity in the inactivator preparation alone was allowed for.

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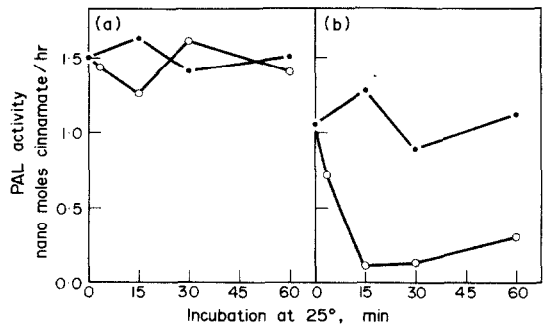

Fig. 2. Effect of cold (4°) treatment on inactivator activity of extract. Inactivator was assayed either (a) immediately after dialysis, or (b) following a 3 hr period at 4° subsequent to dialysis. Solid circles, PAL + buffer; open circles, PAL + inactivator.

No inactivator could be demonstrated in extracts of dark grown material unless the preparation was first held at 4° for 3 hr (Fig. 2). This might imply that the inactivator is present in a bound form (possibly to PAL) in dark tissue.

Partial reversibility of *in vitro* inactivation was observed when the inactivated enzyme was transferred to 37° from 25° and the assays also carried out at the higher temperature (Fig. 3). The reversible nature of the interaction is an indication that the effect is a true inactivation and not proteolysis. Furthermore, we have observed that the inactivator preparation has no effect on the activity of a commercial β-galactosidase preparation indicating the absence of a non-specific protease.

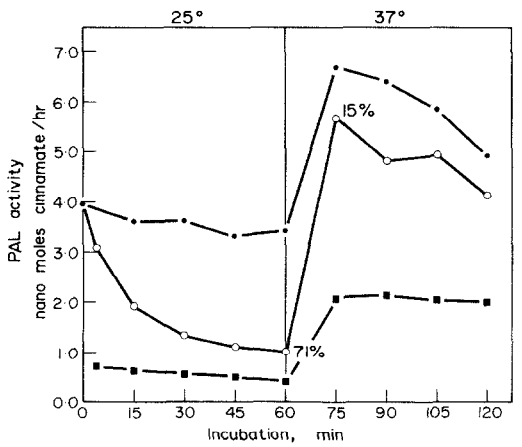

Fig. 3. Effect of transferring inactivated enzyme to 37°. Inactivator (from dark grown plants) was incubated at 25° with PAL from 4 hr blue light treated plants and assayed at time intervals, also at 25°. At T_{60} both PAL + inactivator (open circles), and its control (solid circles), were transferred to 37°. Subsequently, 0·2 ml of reaction mixture was removed and assayed at intervals at 37°. The numbers at T_{60} and T_{75} refer to percentage inhibition of control. Solid squares, PAL activity in the inactivator solution alone. The PAL + inactivator values are corrected for this value.

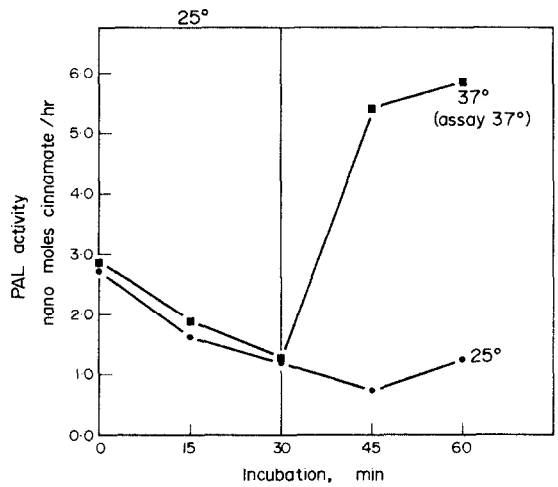

Fig. 4. Effect of transferring a PAL extract from dark grown plants from 25° to 37°. Two samples of PAL extract from dark grown seedlings were incubated at 25°, and 0·2 ml aliquots were withdrawn at intervals for assay at 25°. At T_{30} one sample was transferred to 37° and assays conducted at 37° (solid squares). The remainder was maintained at 25° (solid circles).

The effect of changing the pre-incubation and assay temperature of a PAL extract from dark grown material is shown in Fig. 4. In the first half of the experiment the extract was pre-incubated and assayed at 25°. After 30 min half the extract was transferred to a temperature of 37° and after the indicated periods assayed at 37° for 1 hr. The remainder of the extract was maintained at 25° and assayed at the same temperature. The enzyme activity increased four- to five-fold at the higher temperature. Assuming that the activation energy of PAL is similar to that of most enzyme catalysed reactions (i.e. 10 kcal mol^{-1}) [9] the theoretical stimulation of enzyme activity by assaying at 37° rather than 25° would be 2·1 to 2·2 fold increase. The "extra" activity observed in this experiment can be accounted for if we assume that the enzyme:inactivator interaction is disrupted at the higher temperature (this appears to occur in *in vitro* inactivated enzyme, Fig. 3). Early attempts to demonstrate the presence of an inactivator were conducted at 37° and no inactivation was observed. It appears, therefore, that the enzyme–inactivator complex is unstable at this temperature. The release of "free enzyme" from extracts of

dark grown material by changes in temperature may explain the similar five fold increase in PAL activity in inactivator preparations from light-treated plants (Fig. 3). It is interesting to note that the control preparation, comprising a crude PAL extract from 4 hr blue light-treated plants, conforms to the expected theoretical increase in activity when assayed at the higher temperature. This seems to imply that the control PAL extract consists of "free enzyme" with no inactivator present.

Studies on the kinetics of the enzyme:inactivator relationship showed that a plot of 1/v vs inhibitor concentration (at fixed PAL concentration) approximates to a straight line. Percentage inhibition vs PAL concentration (at fixed concentration of inactivator) is constant over a ten-fold range of enzyme activity. These data indicate that the inactivator:enzyme association is a freely reversible complex [3, 4] and are in agreement with the temperature transfer results.

Studies on some of the properties of the inactivating factor gave the following: the inactivator was stable to a temperature of 85° for 10 min and 60° for 4 hr. Dialysis at 25° for 4 hr (Visking tubing, pore size 2·5 μm, average retention MW 12000) did not remove the inactivating properties of the extract. However, after storage at 4° for several days the inactivator became freely dialysable (Table 1). Furthermore, the PAL activity of the inactivator preparation increased considerably after the second dialysis in correspondence with the decrease in inactivator. The inactivator appears to be of relatively low MW but associated with a larger molecule preventing its exclusion from dialysis tubing at 25°. The association appears to break down at 4° liberating free inactivator which is lost on subsequent dialysis.

The behaviour of the inactivator when precipitated with ammonium sulphate and on columns of Sephadex G-25 is consistent with the idea of a varying association with a high MW "carrier". In some preparations the inactivator was recoverable from 0–80% ammonium sulphate precipitates and on other occasions it was lost on precipitation. When applied to columns of Sephadex G-25 (10 × 1 cm) the inactivator migrated with the front to some extent but was also found in later fractions.

Incubation of the inactivator with RNase and DNase (1 mg/ml for 4 hr at 25°) did not remove the inactivating properties. The inactivator was similarly unaffected by treatment with trypsin and papain. The inactivating factor, therefore, does not appear to be a protein as had previously been suggested [10].

DISCUSSION

The results above are generally in agreement with the suggestion that PAL is present in dark tissue in an inactive form [7]. An inactivator of the enzyme is present in the hypocotyls when the extractable levels of PAL are low (i.e. in dark grown seedlings and those given 12 hr continuous blue light). At the peak of PAL activity caused by blue light treatment (4 hr irradiation [6]), no inactivator appears to be present as evidenced by the correspondence with the expected theoretical stimulation of activity by temperature. In addition, the presence of inactivator at this stage on the time course would be expected to result in a decline in activity in the control PAL extracts when incubated at 25° and this does not occur (Fig. 3). The presence of the inactivator, therefore, appears to correlate with low extractable enzyme levels although more detailed studies on the *in vivo* status

Table 1. Inactivator activity in dialysed preparation after storage, and after redialysis

Treatment	PAL activity* nmoles cinnamate/hr		% Inhibition	
	PAL + Inactivator	Inactivator alone	Uncorrected	Corrected for Inactivator PAL
Stored	1·28	0·31	68	76
Redialysed	4·14	1·60	0	38

* Control = 4·11.

Inactivator preparation was dialysed for 4 hr at 25° and stored at 4° for 3 days (Stored). Inactivator activity was then assayed against PAL and corrected for residual PAL activity in inactivator preparation. A sample of stored inactivator was then redialysed at 4° for 4 hr and immediately assayed against PAL (Redialysed). Percentage inhibition was corrected for residual PAL activity in inactivator preparation.

will be necessary to implicate conclusively the inactivator in the regulation of PAL levels. However, the evidence is consistent with the view that PAL activity in gherkin is controlled by an activation–inactivation mechanism.

EXPERIMENTAL

Gherkin seedlings (*Cucumis sativus* cv. Venlo Pickling) were grown in darkness for four days at 25 ± 1°. The PAL preparation for assay of inactivator was routinely taken from plants treated with blue light (70 W m^{-2}) for 4 hr. Extraction of plant material and assay of PAL were as previously described [6] except that the assay temp. was normally 25°. Inactivator preparations were extracted from dark grown and light-treated seedlings by the following procedure. The upper 2 cm of hypocotyl including the plumular hook was ground in liquid N_2, taken up in 0·1 M borate buffer pH 8·8 and strained through 2 layers of muslin. The extract was centrifuged at 33000 *g* for 5 min. and the supernatant dialysed for 4 hr (1:2000 with stirring). Extracts from dark grown material were maintained at 4° for 24 hr before assay and all inactivator preparations were stored at 4°. The inactivator was stable under these conditions for at least 10 days. Some samples had a residual PAL activity which was allowed for in the results.

Assay of inactivator. One ml of the standard PAL extract was mixed with 1 ml of inactivator and the solution incubated at 25° for certain periods before assaying for PAL activity. A control consisting of 1 ml of standard PAL extract plus 1 ml of buffer was used in all experiments. The PAL activity in the inactivator solution alone was deducted from the PAL plus inactivator value to give the change in activity in the standard PAL extract. This correction factor was seldom of significance (but see Fig. 3) and usually negligible in extracts which had been stored for a few days before use. The pre-incubation period before assay of kinetics was 1 hr.

Treatment with Trypsin and Papain. Solid "Enzygel" preparations of the proteolytic enzymes (obtained from Boehringer-Mannheim, New York) were added to an inactivator preparation to a concentration of 5 mg/ml and incubated at 25° for 4 hr. The enzymes were filtered off as insoluble particles using Whatman GF/A filter paper. The inactivator was assayed against a PAL extract. A control containing buffer instead of inactivator was used to measure any residual proteolytic activity remaining after filtration.

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