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PHENYLALANINE AMMONIA LYASE-INACTIVATING SYSTEM IN SUNFLOWER LEAVES

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Key Word Index—*Helianthus annuus*; Compositae; sunflower; phenylalanine ammonia lyase; inactivation.

Abstract—A high MW fraction extracted from sunflower leaves inactivates the phenylalanine ammonia-lyase (PAL) from sunflower and *Rhodotorula glutinus*. The pH optimum for inactivation was 9·5. The K_m of the phenylalanine ammonia-lyase-inactivating system (PAL-IS) was estimated to be 35 mU PAL/ml. D-phenylalanine was an effective inhibitor of inactivation. Fresh tissue had a low level of PAL-IS, but the level increased upon sucrose treatment with light and was maintained during subsequent treatment of leaves in darkness on water.

INTRODUCTION

Phenylalanine ammonia lyase (PAL) (E.C.4.1.1.5) is the first enzyme unique to the biosynthesis of a large group of plant phenolics. It has been extensively studied and has been shown to undergo rapid changes in activity in plants [1]. The activity of PAL in plants generally increases in response to a variety of stimuli and in some plants subsequently decreases. These frequently rapid fluctuations have been suggested as being important in the regulation of phenolic biosynthesis [2]. The loss of PAL *in vivo* has been reported in a number of different tissues, e.g. potato [3], *Xanthium* [4], gherkin [5, 6], sunflower [7], strawberry [8], etc. [2]. The concept of enzyme turnover is well established in several systems. In mammalian enzymes associated with amino acid metabolism, inactivation was reported to be based on proteolytic activity [9] but the inactivation of potato invertase is associated with a specific protein coupler [10]. The inactivation of apoNAD enzymes of rat is due to a specific inactivating enzyme [11] whereas the turnover of tryptophan synthase of yeast involves a specific proteolytic enzyme [12].

The inactivation of PAL in *Sinapis* was demonstrated to act independently of synthesis [13] and increase with time. An inhibitor of PAL synthesis was reported in gherkin [14] and an *in vitro* inactivator of PAL has been demonstrated in hypotocotyls of this plant. The mechanism was reported to involve a reversible complex formation [15]. We have previously reported the occurrence of a PAL-inactivating system (PAL-IS) in sunflower [2] and now describe some of its properties.

RESULTS

The borate extract of leaves pretreated with 0·1 M sucrose and light (2 mW/cm^2) inactivated added PAL. The PAL-IS activity could be concentrated by ammonium sulfate fractionation. Forty per cent of the PAL-IS activity was precipitated by 45% ammonium sulfate saturation and 100% by 60% saturation. The PAL recovery from borate extracts by ammonium sulfate fractionation was similar to the PAL-IS recovery. The separation of PAL-IS from endogenous PAL was simplified by further pretreatment of leaves on water in darkness following the light/sucrose treatment. This permitted the *in vivo* decay of PAL [7]. The purification sequence shown in Table 1 resulted in a 650 fold purification with a 12% yield. *Rhodotorula glutinus* PAL (estimated MW 270000 [16]) and bovine serum albumin (MW 67000) chromatographed on the same Agarose A-1·5 m column used for PAL-IS were both eluted before the inactivator which appeared at *ca* 65% of the column volume.

The loss of PAL activity with time in the presence of isolated PAL-IS declines logarithmically. (Fig. 1) and results from the dependence on substrate concentration (PAL) at the substrate levels used for assay. The method of Katsunuma *et al.* was used for expressing activity [12] (See Experimental).

The extraction of whole leaves yielded relatively low amounts of PAL-IS so particulate fractions were tested for their inactivation ability (Table 2). Since most of the PAL-IS was found in the pellets obtained in weak centrifugal fields, all subsequent preparations were made as described in the Experimental section.

Table 1. Partial purification of PAL-IS from sunflower leaves

Fraction	Total mU	mU/mg Protein
Extract supernatant	81·4	0·03
45–60% ammonium sulfate pellet	19·2	0·29
Most active fraction from chromatography on Agarose A-1·5 m	10·2	19·7

Leaves were treated for 24 hr with 0·1 M sucrose and 2·0 mW/cm^2 light plus 24 hr on H_2O in darkness. Leaves homogenized in 0·1 M borate (pH 8·8) containing 0·5 mM mercaptoethanol. The homogenized extract was centrifuged (20 000 *g*-20 min) before adding ammonium sulfate to the supernatant. PAL-IS was assayed at 40° with sunflower PAL.

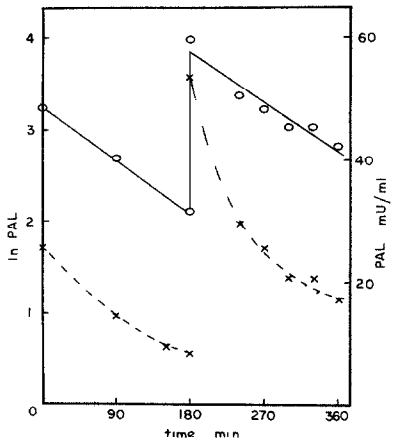

Fig. 1. Loss of sunflower PAL activity. PAL-IS isolated from intact sunflower leaves by ammonium sulfate fractionation (45–80%), Agarose A-1·5 m column and concentration with Amicon Centriflo cones. At 180 min, fresh PAL was added. The PAL activity is plotted as the concentration (×) and the natural logarithm of the concentration (○).

A number of inhibitors were tested using suspended particulate PAL-IS (Table 3). D-phenylalanine was the only effective inhibitor found. The inhibition tests were conducted by adding inhibitor to the PAL/PAL-IS mixture and to PAL alone and assaying PAL activity at 1 hr intervals. Some compounds tested (iodoacetamide, $MnCl_2$, KCN) irreversibly destroyed PAL without added inactivating system.

Only 50% of the PAL-IS could be extracted from the particulate fraction by suspension in water but *ca* 80% was extracted by 1% Tween-20. Precipitation of particulate fractions with acetone, and resuspension in buffer did not yield significant PAL-IS. Chromatography on Agarose A-1·5 m of Tween-20 extracts of the particulate fraction resulted in PAL-IS with a sp. act. of 28 mU PAL-IS/mg protein.

The rate of PAL loss due to PAL-IS was greater using *R. glutinus* PAL than when using sunflower PAL. It was observed that added bovine serum albumin retarded the loss of *R. glutinus* PAL. The inhibition was about 50% for 5 mg/ml BSA. The lower PAL-IS activity when utilizing sunflower PAL was therefore interpreted as being due to its relative impurity.

Table 2. PAL-IS activity of recentrifuged sunflower leaf extracts

Sample	Total mU PAL-IS/fraction
Original suspension	45·8
1000 *g* pellet	14·8
2500 *g* pellet	12·2
5000 *g* pellet	7·2
10000 *g* pellet	2·8
20000 *g* pellet	3·3
20000 *g* supernatant	9·4

Original suspension prepared by extracting leaves with 0·5 M sucrose in 30 mM borate, filtering through 4-layers cheesecloth, centrifuging at 200 *g* for 100 min and then at 17000 *g* for 20 min. The pellet was suspended in 0·5 M sucrose, recentrifuged and the supernatants from each pellet recentrifuged at the next higher force.

Table 3. Effect of some inhibitors on the inactivation of PAL by particulate fractions

Sample	PAL-IS mU/ml
PAL-IS	11·8
Boiled PAL-IS	0·2
PAL-IS + 0·4 mM Phenylmethylsulfonyl fluoride	12·4
PAL-IS + 0·4 mM *O*-phenanthroline	11·8
PAL-IS + 0·2 mM NaN_3	10·6
PAL-IS + 0·2 mM phenazine methosulfate	11·5
PAL-IS + 5·0 mM D-phenylalanine	5·8

PAL-IS prepared by extracting sunflower leaves with 0·5 M sucrose in 30 mM borate (pH 8·8), squeezing through cheesecloth and suspending the pellet between 200 *g* (2 min) and 1000 *g* (10 min).

The pH optimum for sunflower PAL-IS (purified through the Agarose column) was 9·5 in borate, with half maximal activity at pH 7·5. The sunflower PAL-IS inactivation of *R. glutinus* PAL was inhibited 100% by 2·5 mM D-phenylalanine but the inhibition using the less pure sunflower PAL was much less (Table 3). The K_m for partially purified sunflower PAL-IS (through Agarose) was 35 mU/ml PAL (using *R. glutinus* PAL). Assuming that the PAL was pure, the K_m would be 7×10^{-8} M PAL.

The previously reported *in vivo* estimates of PAL-IS suggested that its activity was low in freshly cut leaves, increased upon treatment with 0·1 M sucrose in light and remained high in leaves where the PAL had been allowed to decrease by transfer to water and darkness [7]. The *in vitro* assay of PAL-IS in 1% Tween-20 extracts of the 200–1500 g pellets was 9 mU/g fr. wt for fresh leaves, 18 mU/g fr. wt after 24 hr sucrose/light treatment and 17 mU/g fr. wt following a 24 hr subsequent treatment on water in darkness. The PAL activity of these leaves changed from 11 mU/g fr. wt to 27 mU/g fr. wt during the sucrose/light treatment and decayed to 8 mU/g fr. wt during the subsequent treatment on water in darkness. These *in vitro* assays of PAL-IS correspond to the estimates of activity previously reported *in vivo* [7].

DISCUSSION

The decay of PAL activity *in vivo* has been observed in many different tissues [1]. The *in vitro* loss of PAL activity was associated with a heat-labile extract of sunflower leaves [2] and the presence of a system for inactivating PAL predicted from *in vivo* effects of cycloheximide on the activity of PAL [7]. Similar results with gherkin has resulted in the demonstration of an inactivating fraction in that tissue [15]. The inactivation system reported here requires further purification for study of the specificity and mechanism of action. The PAL-IS described was only observed to inactivate PAL; no predictions can be made whether the action is proteolytic or some form of masking of activity. The protection of PAL from inactivation by D-phenylalanine may be due to protection of the active site from direct attack or to maintainence of the structure which would block the action of the inactivating system as proposed for other turnover systems [9]. The action of inhibitors cannot be fully assessed due to the relative impurity of the system and the sensitivity of the substrate (PAL) to their pres-

ence for the several hours involved in measurement of inactivation.

The PAL-IS was readily purified and stable, its absence in leaves without pretreatment necessitated prior manipulation of tissue before its measurement or isolation. The subcellular distribution in particulates must be further studied to determine the structures containing PAL-IS and studies must be conducted to establish whether this system which functions *in vitro* is of significance in the *in vivo* turnover of PAL.

EXPERIMENTAL

Sunflower plants (*Helianthus annuus*) were grown in the greenhouse or in small growth chambers. *PAL activity* was assayed at 40°, 280 nm in a Beckman DK-2A spectrophotometer using 1 ml 0·6 M L-phenylalanine, sample, and enough 0·1 M borate to make a final vol. of 3 ml. A unit (U) is defined as the production of 1 μmol cinnamic acid/min at 40°.

Phenylalanine ammonia-lyase sources. PAL was partially purified from sunflower leaves floated on 0·1 M sucrose in the light (2 mW/cm^2), by extraction with 0·1 M borate (pH 8·8) containing 0·5 mM mercaptoethanol. This extract was subjected to $(NH_4)_2SO_4$ fractionation and the ppt between 45 and 60% saturation applied to an Agarose A-1·5 m column. The most active fractions were collected, concentrated with Amicon Centriflo cones (Amicon Corp. Lexinton, Mass.) and frozen for storage. PAL from *Rhodotorula glutinus* was purchased from P-L Biochemicals (Milwaukee, Wisc.) and subjected first to chromatography on an Agarose A-1·5 m column and then following concentration of the most active fractions to an A-15 m column. Several fractions had a sp. act. of 1·8 U/mg which were frozen and used as substrate for the inactivating system. *PAL-IS activity* was assayed by adding known amounts of PAL (sunflower or *R. glutinus*) to borate buffer containing the extract to be assayed. Samples were withdrawn at intervals (usually 30 min or 1 hr) and the PAL activity assayed. Controls were made of PAL in buffer alone. PAL activity was lost slowly from controls. The loss was linear and much slower than the loss of activity in the presence of added PAL-IS. When using sunflower PAL as substrate, 40° could be used for inactivation assay because the loss of PAL activity in controls was not significant. *R. glutinus* PAL was not stable in controls at 40° and 30° was used for assays using this source. The inactivation of sunflower PAL by PAL-IS was twice as great at 40° than at 30°.

PAL-IS units. The method of Ref. [12] was used in expressing the activity of PAL-IS. A unit is defined as: $\ln(E)_0 - \ln(E)_t \times t^{-1}$ where $(E)_0$ is the PAL activity at time zero and $(E)_t$ the activity at t min of exposure to PAL-IS. The activity of PAL was determined at several times after t_0, the ln PAL plotted vs time, the least squares line calculated and the slope expressed as units. The units are therefore dependent on concentration of PAL and only relative comparisons can be made between experiments.

Preparation of enzyme for PAL-IS assay or purification. Sunflower leaves were homogenized in 0·4 M Sucrose in 30 mM Borate for 30 sec (Virtis homogenizer), the homogenate squeezed through 4-layers of cheesecloth, centrifuged at 200 *g* for 2 min, the pellet discarded, and the supernatant centrifuged at 1500 *g* for 10 min. The pellet was collected, stirred with 1% Tween-20 for 30 min, centrifuged at 15 000 *g* for 30 min. The clear supernatant was used as enzyme in the PAL-IS assay, or was applied to an Agarose A-1·5 m column for further purification and eluted with 30 mM borate pH 8·8 containing 0·5 mM 2-mercaptoethanol. The PAL-IS preparations could be stored at −10° if rapidly frozen in liquid N_2 but lost activity if frozen slowly or refrozen several times.

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