

Use of Immunotitration to Demonstrate Phytochrome-Mediated Synthesis *de novo* of Chalcone Synthase and Phenylalanine Ammonia Lyase in Mustard Seedling Cotyledons

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Sinapis alba, Enzyme Induction by Phytochrome, Immunotitration, Chalcone Synthase, Phenylalanine Ammonia Lyase

The suggestion [H. J. Newbury and H. Smith, Eur. J. Biochem. **117**, 575–580 (1981)] of a posttranslational activation of accumulated enzyme precursor by phytochrome was tested in the case of phytochrome-mediated induction of chalcone synthase (EC 2.3.1.74) in mustard seedlings. The approach of immunotitration of enzyme activity was adopted since it allows to relate the amount of antigenic material present in a particular extract to the extract's enzymatic activity. The data obtained show that under all experimental conditions enzyme activity of an extract is proportional to the amount of immunoresponsive material. No indication was found either of light-dependent modification of the enzyme or of enzymatically inactive immunoresponsive material in extracts prepared from dark-grown mustard seedlings or from light-treated seedlings placed again in darkness. In model experiments with purified ribulose-bisphosphate carboxylase (EC 4.1.1.39) heat-inactivated enzyme was detected by the antibodies. Experiments on induction of phenylalanine ammonia lyase (EC 4.3.1.5) by phytochrome in mustard cotyledons support the result – synthesis *de novo* of enzyme protein – obtained previously [W. Tong and P. Schopfer, Proc. Nat. Acad. Sci. U.S.A. **43**, 4017–4021 (1976)] with the technique of density labelling. It is concluded that the phytochrome-mediated induction of enzymes involved in anthocyanin formation in mustard cotyledons is due to synthesis *de novo* of enzyme protein.

Introduction

In seedlings of higher plants as well as in cell suspension cultures derived from higher plants synthesis of flavonoid compounds – anthocyanins in seedlings [1], flavone and flavonol glycosides in cell cultures [2] – is induced by light *via* phytochrome. The production of flavonoids is preceded by the induction of enzymes of the phenylpropanoid and flavonoid pathways among which PAL and CHS have been particularly well investigated [3–5]. Hahlbrock's group has shown unambiguously that in cell suspension cultures of parsley (*Petroselinum hortense*) light-mediated increases in the activity of CHS and PAL are due to

synthesis *de novo* of these enzymes. The enzyme induction by light has been attributed to increased rates of transcription of the pertinent genes [6, 7]. In seedlings of higher plants, however, the situation is less clear [8]. While in the case of PAL in mustard cotyledons, Tong and Schopfer [9] have shown unambiguously – using density labelling – “that phytochrome-mediated PAL accumulation involves a stimulation of synthesis *de novo* of enzyme protein” rather than light activation of pre-existing pro-enzyme of PAL [10, 11], the application of immunological techniques to phytochrome-mediated induction of enzyme activity has led to conflicting results. As a recent example, Newbury and Smith [12] used purified antiserum – raised against *Curcubita* ascorbate oxidase and cross-reacting with the mustard enzyme – to measure by rocket immunoelectrophoresis the amount of ascorbate oxidase protein in extracts from mustard cotyledons. They confirmed that after transfer from darkness to far red light (operating *via* phytochrome) the activity of the enzyme in mustard cotyledons strongly increased [13], but they found that the amount of enzyme protein remained the same. Unimbibed mustard seeds were shown to contain the same amount of antigenic protein as expanding coty-

Abbreviations: CHS, chalcone synthase (EC 2.3.1.74); PAL, phenylalanine ammonia lyase (EC 4.3.1.5); Carboxylase, ribulose-bisphosphate carboxylase (EC 4.1.1.39); FR, far red light; P_{fr} , far red light absorbing form of the phytochrome system; P_r , red light absorbing form of the phytochrome system; ϕ , photoequilibrium of phytochrome (P_{fr}/P_{tot}).

Reprint requests to Prof. H. Mohr.

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ledons although no enzymatic activity could be extracted from the seeds. This result conflicts with previous reports on phytochrome-mediated induction of ascorbate oxidase activity in mustard cotyledons which were based on density labelling methods. Acton *et al.* [14] and Attridge *et al.* [10] had found that far red light led to an increase in the rate of synthesis of ascorbate oxidase. The apparent inconsistency of immunological and density labelling data prompted us to reinvestigate whether activation of pre-existing pro-enzyme plays in fact a role in phytochrome-mediated induction of enzyme activity.

In the present study the method of immunotitration of enzyme activity was adopted since it allows to relate the amounts of antigenic material present in a particular extract to the extract's enzymatic activity. Moreover, the method allows to detect such changes of the enzyme molecule which affect its binding to the antibodies. Our work concentrates on CHS in mustard cotyledons since no information on synthesis *de novo* vs. activation of pro-enzyme was available so far with this (probably rate-limiting) [5] enzyme of the flavonoid pathway. PAL was also included in the study to test whether or not immunotitration would confirm the data obtained by Tong and Schopfer [9] with the density labelling technique (see above).

Materials and Methods

Seeds of white mustard (*Sinapis alba* L., harvest 1979), purchased from Asgrow Company (Freiburg-Ebnet, FRG) were selected and the seedlings were grown at 25 ± 0.5 °C according to the previously described procedure [15].

Standard far red light (FR, 3.5 W m^{-2} , $\varphi_{\text{FR}} = 0.025$) and long wavelength far red light (RG9-light, 10 W m^{-2} , $\varphi_{\text{RG9}} < 0.01$) were obtained as described previously [16].

For preparation of the crude extract, twenty pairs of cotyledons were ground for 3 min with 1.5 g quartz sand, 0.3 g Dowex 1X2 and 6 cm³ of 0.1 M potassium phosphate buffer, pH 7.5, containing 2 mM mercaptoethanol. The homogenate was centrifuged at $35,000 \cdot g$ for 20 min. For PAL and CHS assays, aliquots of the supernatant below the fat layer were passed through a Sephadex G-25 column. For carboxylase assay the supernatant was directly used.

For the isolation of carboxylase, 160 pairs of mustard cotyledons were ground with 10 cm³ of extrac-

tion buffer (0.1 M TRIS/HCl buffer, pH 7.8, 0.02 M MgCl₂). The native enzyme was isolated by sucrose gradient centrifugation as described by Goldthwaite and Bogorad [17]. The purity of carboxylase was determined in a sodium dodecyl sulfate polyacryl gel electrophoresis where no other bands beside the small and the large subunit of the carboxylase could be found after Coomassie blue staining (this analysis was kindly performed by R. Oelmüller).

Enzyme assays. Carboxylase was assayed as described elsewhere [18]. CHS was assayed in principle after Kreuzaler and Hahlbrock [19]. The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 10 µM *p*-coumaroyl coenzyme A, 5 µM [2-¹⁴C]malonyl coenzyme A (2.15 GBq mmol⁻¹), and extract (20% of the total assay volume, 0.1 cm³). The assay was performed at 30 °C for 20 min. The reaction was stopped with 0.02 cm³ of 1 mg cm⁻³ unlabelled naringenin solution. Total naringenin was extracted with ethyl acetate, separated by thin layer cellulose chromatography, and the amount of labelled naringenin was determined in a liquid scintillation counter. PAL activity was assayed after Betz [20]. The assay mixture contained 0.1 M borate buffer, pH 8.8, 30 µM L-[U-¹⁴C]phenylalanine (6.0 GBq mmol⁻¹) and extract (80% of total assay volume, 0.05 cm³). The assay was incubated for 40 min at 25 °C and the reaction was stopped with 0.01 cm³ of 2.5% trichloroacetic acid. Then 0.01 cm³ of 1 mg cm⁻³ trans cinnamic acid in MeOH was added as a marker. Total *trans* cinnamic acid was extracted by diethyl ether, separated by thin layer cellulose chromatography, and the amount of label in *trans* cinnamic acid was determined in a liquid scintillation counter.

Polyvalent antisera raised against CHS and PAL from parsley cell suspension cultures but strongly cross reacting with the mustard enzymes were a gift of Prof. K. Hahlbrock [21]. The antiserum against native carboxylase was raised in rabbits by R. Oelmüller in our laboratory. As a rule, precursor forms are recognized by antibodies raised against native (see [22], for review).

For immunotitration of enzyme activity increasing amounts of antiserum were added to a particular enzyme extract in an assay mixture which contained 0.15 mM NaCl and a constant amount of non-immune control serum. (Carboxylase: 0.005 cm³ non-immune serum/assay; CHS: 0.02 cm³/pair of cotyledons; PAL: 0.02 cm³/pair of cotyledons). The assays

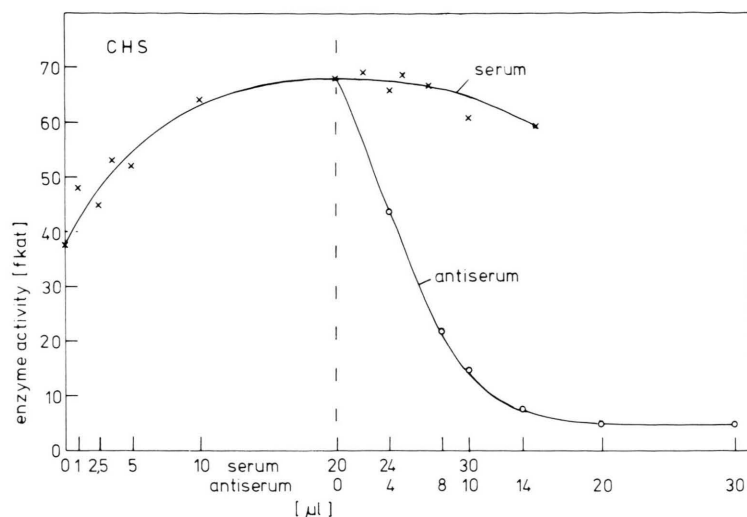


Fig. 1. The effect of non-immune control serum and antiserum on CHS activity. Results shown are per cotyledon pair.

were incubated for 10 min at 25 °C and then for 20 min at 4 °C. Then the enzyme reaction was started by adding the appropriate substrates and enzyme activity was determined as described above.

The addition of a saturating amount of non-immune control serum (Fig. 1) is an essential step in immunotitration in the case of CHS and carboxylase to account for a non-specific *stimulatory* effect of the serum on enzyme activity. The non-specific action of serum and the specific inhibitory effect are obviously superimposed (Fig. 1).

Statistics. The enzyme activities shown in Table I are mean values (\pm estimated standard errors) of at least 8 independent measurements. The assays contributing to a particular immunotitration curve were performed in duplicate.

Results

Model experiments with carboxylase

The principal usefulness of immunotitration is shown in Fig. 2 using different dilutions of purified carboxylase extracted from mustard cotyledons and antiserum raised against the purified enzyme. For a particular immunotitration curve different amounts of antiserum are given to the assay mixture which always contained the same amount of extract. The dashed lines in Fig. 2 show that the amount of antiserum required to reduce enzyme activity by a certain percentage (25, 40, 50%) is directly proportional to the original enzyme activity. This means that the enzyme activity of an extract – without antiserum – is proportional to the amount of immunoresponsive material in that extract.

Table I. Levels of enzyme activities of PAL and CHS in extracts of cotyledons from differently treated mustard seedlings ($n = 8$).

| Treatment | PAL | CHS |
|----------------------------|--|--|
| | $\left[\frac{\text{fkat}}{\text{pair of cotyledons}} \right]$ | $\left[\frac{\text{fkat}}{\text{pair of cotyledons}} \right]$ |
| 36 h dark (D) | 22 ± 1 | 0.4 ± 0.03 |
| 36 h D + 6 h FR | 173 ± 10 | 21.7 ± 1.0 |
| 36 h D + 18 h FR | 485 ± 14 | 71.6 ± 2.5 |
| 36 h D + 36 h FR | 143 ± 11 | 42.2 ± 2.0 |
| 36 h D + 12 h FR | | |
| + 10 min RG9-light + 6 h D | 220 ± 7 | 58.1 ± 2.3 |
| 36 h D + 36 h D | 17 ± 1 | 0.7 ± 0.03 |

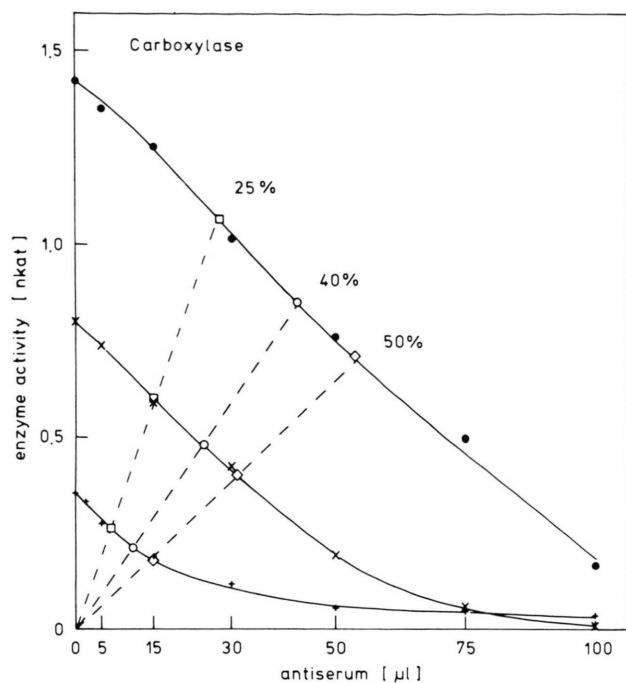


Fig. 2. Immunotitration of purified native carboxylase extracted from mustard cotyledons and differently diluted. Non-immune control serum had no inactivating effect. The dashed lines show that the amount of antiserum required to reduce original enzyme activity by 25, 40 or 50% is directly proportional to the original activity.

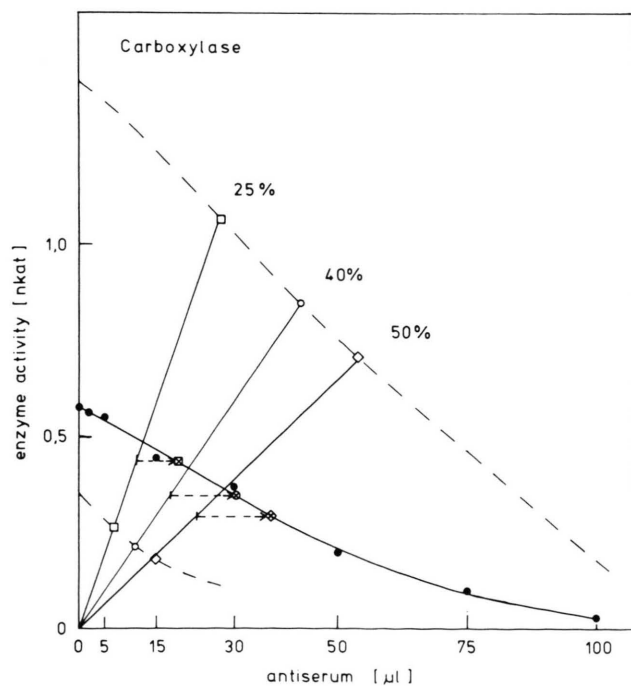


Fig. 3. Immunotitration of a mixture of purified native and heat-inactivated carboxylase extracts. The dashed lines (same as in Fig. 2) show the immunotitration curves without inactive enzyme. If heat-inactivated enzyme (same amount as active enzyme) is added to the native enzyme the requirement for antiserum to obtain a certain reduction of enzyme activity is increased (\boxtimes , \otimes , \boxtimes).

If heat-inactivated carboxylase is added to the solution of active enzyme the requirement for antiserum to obtain a certain reduction of activity is correspondingly increased (Fig. 3, horizontal arrows). This shows that the antiserum is able to detect enzymically inactive carboxylase. Under these circumstances proportionality between enzyme activity and amount of immunoresponsive material (solid line) does no longer exist.

Goal of the present study

On the basis of this model study with carboxylase we pose the question of whether any pro-enzyme (not yet activated) and/or breakdown product (enzyme which has been active and was then inactivated prior to degradation) can be distinguished in cotyledon extracts from differently treated mustard seedlings by antibodies directed against CHS and PAL. If we obtain the same results as shown in Fig. 2 (proportionality between enzymatic activity and amount of immunoresponsive material) we may conclude that the immunoresponsive activity per unit of active enzyme does not change with different light treatments. This would permit the conclusion that post-translational activation (if it occurred!) was very rapid compared with the time for synthesis *de novo* of the pro-enzyme. Correspondingly, we could conclude that degradation of the enzyme protein was so fast

that any inactivated enzyme prior to degradation would not accumulate.

Experiments with CHS

Table I shows the levels of CHS activity extracted from differently treated mustard seedlings. Immunotitrations of CHS in extracts from these differently treated mustard seedling cotyledons indicate (Fig. 4) that under all experimental conditions tested (including mixing experiments) enzyme activity of each extract is strictly proportional to the amount of immunoresponsive material.

Experiments with PAL

Table I shows the levels of PAL activity extracted from differently treated mustard seedlings. Immunotitrations of PAL activity in extracts from these differently treated mustard seedling cotyledons show indeed (Fig. 5) that enzyme activity correlates with the amount of immunoresponsive material. However, the dashed lines do not extrapolate to zero as in the case of CHS (see Fig. 4). If PAL is immunotitrated in differently diluted extracts from any particular seedling stage the same phenomenon is observed (Fig. 6): the points for 25, 40, 50 and 60% reduction of enzyme activity are again on a straight line, and the lines cross at a single point (the same

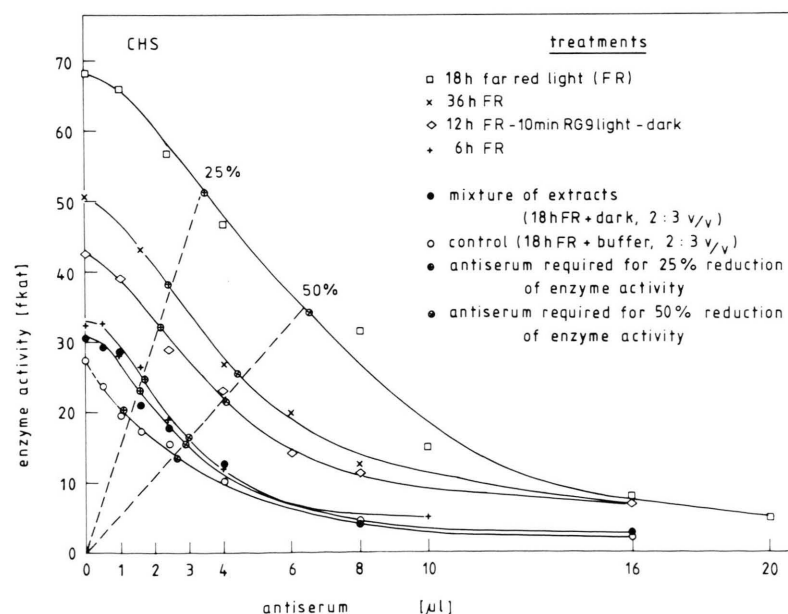


Fig. 4. Immunotitration of CHS in extracts from mustard cotyledons. Non-immune control serum had no inactivating effect (see Fig. 1). The column headed "treatments" designates the treatment of the mixed extracts to test for immunoresponsive material in an extract from dark-grown seedlings. The dashed lines show that the amount of antiserum required to reduce enzyme activity by 25 or 50% is directly proportional to the activity of the original extract. Results shown are per cotyledon pair.

point as obtained in immunotitrations of extracts from differently treated cotyledons, see Fig. 5), but this is not point zero. Since dilution does not change enzyme activity per unit enzyme, interpretation of the dilution effect is not clear at present. Irrespective of the interpretation of this anomaly (see Discussion), the basic message of Fig. 5 is straightforward in the case of PAL as well: There is a linear correlation between enzyme activity in the original extract and the amount of immunoresponsive material in that extract.

Discussion

Immunotitration of enzyme activity is an elegant method to test for proportionality between enzymatic activity and amount of immunoresponsive material. The data obtained with CHS from mustard seedling cotyledons indicate that under all experimental conditions enzyme activity of an extract is proportional to the amount of immunoresponsive material. It is concluded from this observation that

neither pro-enzyme (synthesized but not yet activated) nor breakdown products (enzyme which had been active and was inactivated prior to degradation) existed in the extracts which could be detected by antibodies raised against the active enzymes. These results agree with the conclusions drawn by Reimold *et al.* [23] that CHS in parsley cell suspension cultures is apparently neither cotranslationally nor posttranslationally modified. This previous conclusion was based on a comparison of the nucleotide sequence of CHS mRNA and of the amino acid sequence of the enzyme.

Regarding PAL, our data are compatible with the results obtained by Tong and Schopfer [9] with the density labelling technique that phytochrome-mediated induction of PAL activity is due to synthesis *de novo* of enzyme protein. However, the anomaly observed with PAL (Figs 5, 6) shows the limitations of quantitative immunological approaches. As long as the data agree with the theoretical expectation (see Fig. 2) the interpretation is straightforward; however, when deviations appear — such as in the case of

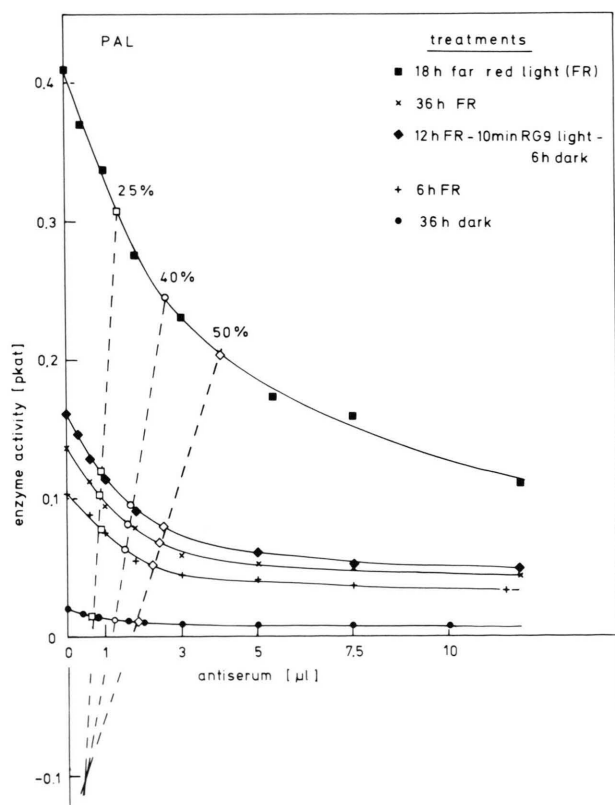


Fig. 5. Immunotitration of PAL in extracts from differently treated mustard cotyledons. Non-immune control serum had no effect. The column headed "treatments" designates the treatment of the seedling before extraction (see Table I). The dashed lines indicate the amount of antiserum required to reduce enzyme activity by 25, 40 or 50%. Results shown are per cotyledon pair.

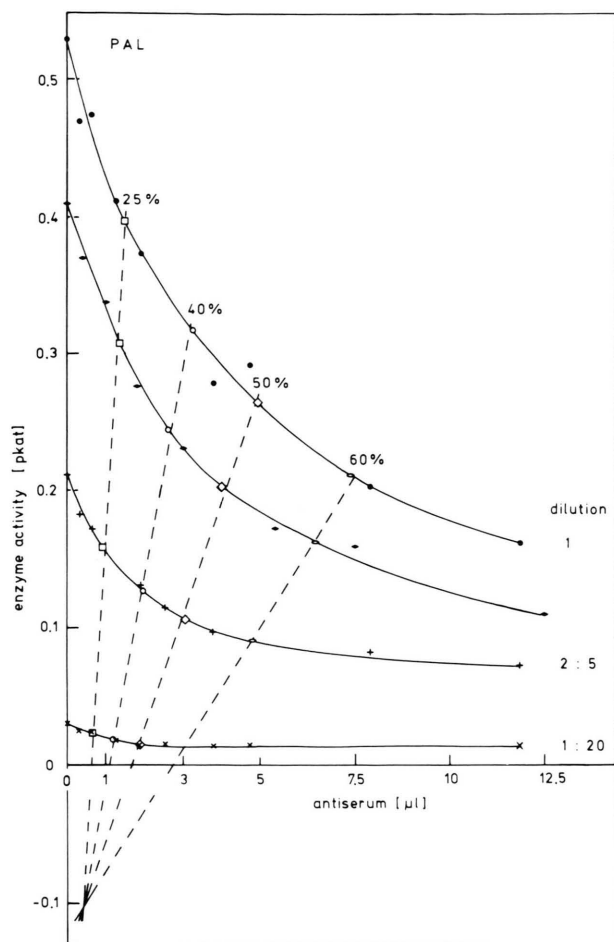


Fig. 6. Immunotitration of PAL in differently diluted extracts from mustard cotyledons. The seedlings were kept in darkness for 36 h and then in far red light for 18 h. ♦, extract from identically treated seedlings but prepared differently. Enzyme was extracted from 80 pairs of cotyledons (instead of usual 20) and diluted before immunotitration.

PAL – interpretation of the data remains ambiguous as long as the nature of the interaction between antibody and enzyme is unknown. Since the titration curves differ conspicuously between carboxylase/CHS and PAL, corresponding differences in the mechanism of interaction between enzyme and antibody must be expected. An explanation of the anomaly observed with PAL is that the enzyme – antibody complex shows some residual enzymatic activity. This is suggested by the titration curves in Figs. 5, 6. This explanation could be tested by a two – stage procedure of immunotitration as described by Dunnette and Weinshilboum [24]. Shortage of PAL antibodies did not allow to perform these experiments in the present study.

Recent data on enzymes of the nitrate assimilation pathway, obtained with immunological methods in several laboratories, support the view that light-mediated appearance of enzyme activity in plant material during photomorphogenesis is due to synthesis *de novo* of enzyme protein: The increase in nitrite reductase activity following exposure of dark-grown wheat plants to nitrate and light was shown by immunodecoration of Western blots to be due to synthesis *de novo* [25]. In the case of nitrate reductase, a high correlation between the extractable enzyme activity and immunochemically (Western blot) assayable nitrate reductase was demonstrated [26]. Increase of chloroplastic glutamine synthase during greening of etiolated rice leaves was found to be due

to synthesis *de novo* of enzyme protein by immuno-precipitation and protein measurement in sodium dodecyl sulfate polyacrylamide gels [27].

Thus it seems that presently available evidence does not support the view that 'light activation of pro-enzymes' plays a role in light-mediated increase of enzyme activity during photomorphogenesis.

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