IDENTITY OF DIFFERENTLY-INDUCED PHENYLALANINE AMMONIA-LYASES FROM CELL SUSPENSION CULTURES OF *PETROSELINUM HORTENSE*

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1. Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the formation of *trans*-cinnamate from L-phenylalanine. This is the first reaction in a sequence of steps involved in the biosynthesis of phenylpropanoid compounds in parsley (*Petroselinum hortense* Hoffm.). Upon irradiation of the cells with ultraviolet light, PAL is induced concomitantly with the other enzymes of general phenylpropanoid metabolism and, in addition, the enzymes of the flavonoid glycoside pathway [1]. When the cell cultures are diluted into fresh medium or water, the enzymes of general phenylpropanoid metabolism, but not of the flavonoid glycoside pathway, are induced [2,3].

Either irradiation or dilution of the cell cultures can be used to induce large changes in the amount of translatable polyribosomal PAL mRNA [4,5]. An antiserum directed against light-induced PAL has been used for immunoprecipitation of PAL subunits in the mRNA translation assay in vitro. The serum reacted equally well with light-induced and dilution-induced PAL, and a double-diffusion assay gave no indication for differences in the binding affinities of the two enzymes for the antibody [5].

However, further investigations at the mRNA level require a more rigorous comparison of the two differently induced enzymes. Here we show that their tryptic peptides are identical, indicating that the same mRNA is involved in their synthesis. In addition, the specificity of the antiserum for PAL species from 3 other sources was investigated.

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2. Experimental

2.1. *Enzymes*

The methods of propagation and harvest of parsley cell suspension cultures have been described [6]. All experiments were performed with day 7, dark-grown cultures whose medium had a conductivity of 1.3–1.5 mmho [7]. PAL was induced either by irradiation of previously dark-grown cells [6] or in the absence of light by dilution of cell cultures into water [3]. Light-induced, radioactive PAL from parsley cells was isolated from 40 ml cell suspension culture which had been continuously irradiated for 8 h and labeled with 200 μCi of a amino [^{14}C] acid mixture (New England Nuclear, Boston, MA) for an additional period of 1 h. Dilution-induced, radioactive PAL was prepared from 6 g cells (fresh wt) which had been propagated for 5 h in 1.2 l sterile water and then labeled for an additional 2 h under the same conditions with 1.6 mCi of a [^{3}H] amino acid mixture (New England Nuclear, Boston, MA). Immunoprecipitation of the labeled enzymes was performed as in [5]. The enzyme subunits were isolated by disc gel electrophoresis in the presence of sodium dodecyl sulfate [4,5] and then precipitated from the resulting solution (3 ml each) by adding 1.5 mg albumin and 750 μl 50% trichloroacetic acid. After standing for 12 h at 4°C, the mixtures were centrifuged at 3000 × *g* for 5 min. The pellets were washed twice with ice-cold acetone and dissolved in 1.2 ml 0.2 M NH_4HCO_3 (pH 8.5). The yield was 0.65 × 10^6 cpm ^{14}C-labeled and 1.5 × 10^6 cpm ^{3}H-labeled PAL subunits.

Cell suspension cultures of soybean (*Glycine max* L.) were grown in the dark. Cell growth was monitored

by measuring the conductivity of the medium, and cells were harvested at the beginning of the progressive deceleration phase of culture growth at a conductivity of ~1 mmho, which represents the peak in PAL activity [8].

Approximately 14-fold purified PAL from cell suspension cultures of carrot (*Daucus carota* L.) was a kind gift from Dr Seitz, Tübingen.

PAL from the yeast, *Rhodotorula glutinis*, was purchased from P-L Biochemicals, Milwaukee, WI. The enzyme preparation was suspended without further purification in a 60% saturated $(NH_4)_2SO_4$ solution (PAL act. 167 nkat/ml, 54 mg protein/ml). This suspension was diluted 1:200 with 0.1 M sodium borate (pH 8.8).

2.2. *Antiserum*

The serum described in [5] was used without further purification.

2.3. *Tryptic peptides*

For tryptic digestion, 3×10^5 cpm of ^{14}C-labeled and 7.5×10^5 cpm of ^{3}H-labeled PAL subunits were combined to give a solution of 1 ml containing 1.5 mg albumin and ~0.1 mg PAL. The protein was partially digested at 37°C for 5 h with 2 additions of 40 μg trypsin (dissolved in 40 μl 1 mM HCl). The mixture was freeze-dried, redissolved in 1 ml 0.1 M pyridine acetate (pH 2.5) and the peptides were separated on an Aminex A-5 column [9]. Fractions of 1.65 ml were collected and dried at 80°C. The residue was dissolved in 1 ml water and evaporated again. After addition of 0.7 ml water and 5 ml of a mixture of toluene and Triton X-100 (2:1, v/v) containing 6 g PPO/l, radioactivity was measured by scintillation spectrometry.

3. Results and discussion

An analysis of the differently labeled tryptic peptides from light-induced and dilution-induced PAL species from parsley cells on an Aminex A-5 column is shown in fig.1. No difference between the two radioactivity profiles was observed, indicating that the two enzyme preparations were very similar or identical with respect to the primary structure of the proteins. This suggests that the same mRNA is

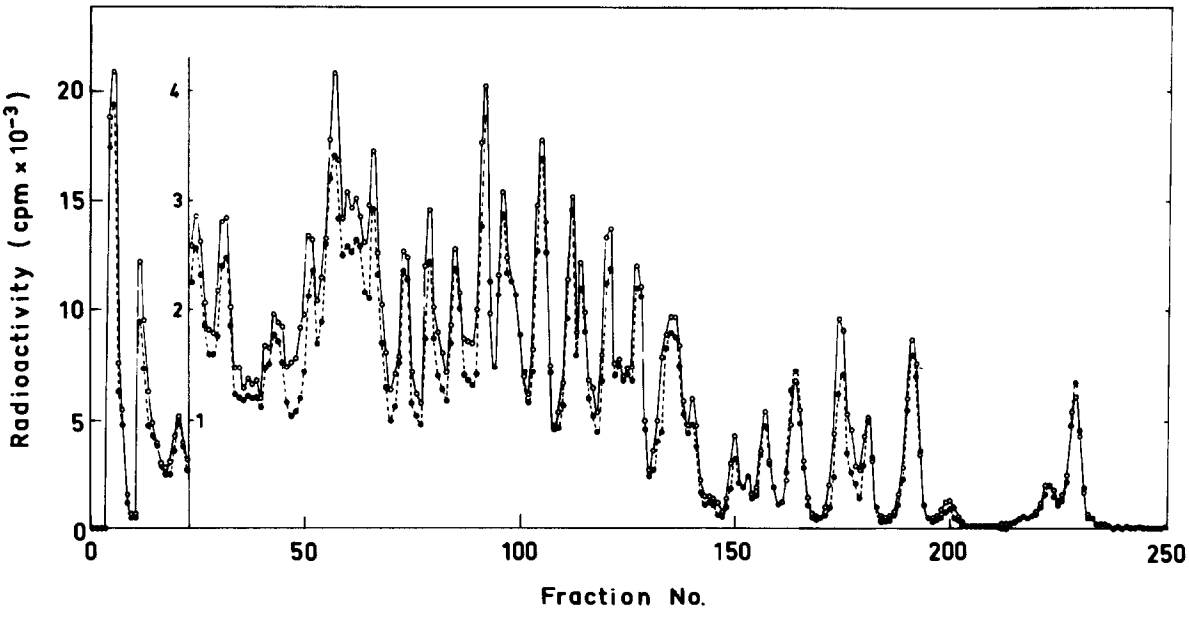

Fig.1. Analysis of tryptic digests from light-induced, ^{14}C-labelled PAL (○) and dilution-induced, ^{3}H-labelled PAL (●) on an Aminex A-5 column. Data shown were normalized to cpm/fraction (○) and cpm/0.4 parts of a fraction (●).

involved in the two cases of induced PAL synthesis. For two reasons, this result was not unexpected and confirmed a previous assumption.

(1) The patterns of initial increases in mRNA as well as in enzyme activity were almost identical under both conditions of induction, including an apparent lag of ~2 h which preceded the increase in enzyme activity [2,3,5].
(2) The combined effects of irradiation and dilution were apparently synergistic [5], suggesting the existence of a common step in the two mechanisms of enzyme induction.

An important practical aspect of the present finding is the possibility of isolating relatively large amounts of the same PAL or PAL mRNA from simultaneously diluted and irradiated cultures, as compared with the amounts obtainable from cultures which had received only 1 of the 2 treatments (cf. [5]). This is likely to apply also to the 2 other enzymes (and their mRNAs) of general phenylpropanoid metabolism, i.e., cinnamate 4-hydroxylase and 4-coumarate:CoA ligase.

The rising interest in the mechanism of regulation of PAL activity in many different plant species has prompted us to investigate the possibility of using the antiserum directed against the parsley enzyme for enzymes from other sources as well. For this purpose, the antiserum was reacted with PAL preparations from another Umbellifera, carrot (*Daucus carota*), from a Leguminosa, soybean (*Glycine max*), and from a microorganism, the yeast *Rhodotorula glutinis*. A double-diffusion assay (fig.2) shows that efficient precipitation was observed for the enzymes from parsley, carrot and soybean, whereas no precipitation of the enzyme from *Rhodotorula* was detectable. Similarly, an antiserum to PAL from mustard (*Sinapis alba*) failed to give detectable precipitin lines with *Rhodotorula* PAL (S. Gupta and G. J. Acton, personal communication).

As in [5], the precipitin lines of light-induced and dilution-induced PAL from parsley cells were connected by smooth curves, a result which is in agreement with their apparent structural identity. The precipitin lines formed by the enzymes from parsley and carrot were also connected by smooth curves. In contrast, a spur occurred, when parsley and soybean PAL were placed in neighbouring wells (fig.2), and a weaker spur was detected between the enzymes from carrot and soybean. In both cases, the spurs pointed towards the well containing soybean PAL.

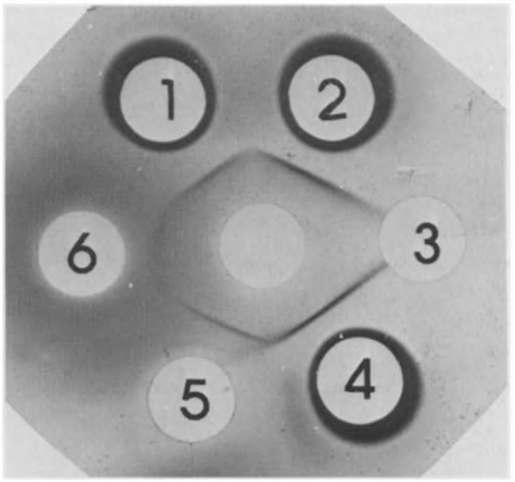

Fig.2. Double-diffusion assay of PAL preparations from different sources. The center well contained 20 μl of the antiserum (diluted 1:10 with 150 mM NaCl). The peripheral wells contained enzyme preparations from (1) and (4) irradiated parsley cells (55 μg protein), (2) diluted parsley cells (100 μg protein), (3) *Rhodotorula* (60 μg protein), (5) soybean (60 μg protein), (6) carrot (130 μg protein). All enzyme solutions contained 50 mM Tris–HCl (pH 7.5) and 100 mM NaCl in 20 μl total vol. The weak reaction of carrot PAL in this particular experiment was paralleled by a considerably reduced enzyme activity after freezing and thawing within a few days.

The activities of the enzymes from all 4 sources investigated were efficiently inhibited by the antiserum for parsley PAL. The following amounts of undiluted antiserum were required for a 50% inhibition of 20 pkat of PAL activity in 100 μl: 1.5 μl for parsley (either light-induced or dilution-induced); 0.2 μl for carrot; 2.5 μl for soybean; 0.2 μl for *Rhodotorula* PAL. It is especially interesting to note the very efficient inhibition of PAL activity from *Rhodotorula*, despite the apparent failure of the antiserum to precipitate this enzyme.

These results suggest that all 4 PAL species have similar or identical antigenic determinants related to their catalytic activities, while other antigenic determinants could be species-specific. It is therefore possible that any PAL antiserum could be used to inhibit this enzyme activity from most or all plants,

while efficient immunoprecipitation might be achieved only with PAL antisera for species of sufficient phylogenetic relationship.

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