Studies on the Regulatory Role of *trans*-Cinnamic Acid on the Activity of the Phenylalanine Ammonia-Lyase (PAL) in Suspension Cultures of *Daucus carota L.*

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In vivo and *in vitro* experiments were performed in order to study the regulatory role of *trans*-cinnamic acid and its hydroxylated derivatives (*p*-coumaric acid, caffeic acid) on the deamination of phenylalanine catalyzed by PAL (EC 4.3.1.5). *Trans*-cinnamic acid inhibits growth and reduces the content of soluble proteins of anthocyanin-containing carrot cells grown in suspension. There is strong evidence from the polysomal patterns and from the effect of *trans*-cinnamic acid on protein synthesis *in vitro* that protein synthesis is inhibited. The kinetic data of PAL clearly demonstrate that *trans*-cinnamic acid inhibits the enzyme by a noncompetitive mechanism. On the contrary, L-α-aminooxy-β-phenylpropionic acid (L-AOPP), a competitive inhibitor of PAL, does not affect protein metabolism.

Introduction

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) which catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid, has an important role in the regulation of the general phenylpropanoid pathway [1]. *Trans*-cinnamic acid is thought to affect the activity of PAL by negative and positive mechanisms: Whereas Engelsma [2] described an inhibition of the extractable enzyme activity by *trans*-cinnamic acid and its hydroxylated derivatives in several plant systems, Dixon *et al.* [3] were able to show a modulation of the extractable PAL activity in *Phaseolus vulgaris* suspension cultures by feedback and feed-forward effects of that pathway intermediates, possibly by enzyme stabilization. More recently, Shields *et al.* revealed a specific "dual control" of the PAL activity over both production and removal of the enzyme by *trans*-cinnamic acid in pea epicotyls [4].

Rapidly growing, anthocyanin-containing suspension cultures of *Daucus carota* are a suitable system for an investigation of specific effects of the pathway intermediates, since their interaction with cellular metabolism and the effect on growth is possible to be recorded over longer periods of cultivation under sterile conditions. In a previous paper we were able to demonstrate that *trans*-cinnamic acid could not compensate for the inhibition of anthocyanin synthesis, caused by L-α-aminooxy-β-phenylpropionic acid (L-AOPP) [5], as described for buckwheat hypokotyls [6]. The inhibition of partially purified PAL *in vitro* occurs only at very high concentrations of *trans*-cinnamic acid. The K_I was calculated to be 1.8×10^{-4} M [5]. In the present communication experiments are described which were able to reveal whether the inhibition of PAL by *trans*-cinnamic acid is specific. Therefore growth and protein content of the cells were determined after application of *trans*-cinnamic acid and L-AOPP. In order to obtain an insight into the protein synthesis capacity *in vivo*, polysomes were extracted from cells treated with *trans*-cinnamic acid and separated on linear sucrose gradients. The ratio of monosomes to polysomes was used as an indication for the protein synthesis *in vivo* [7]. In a further series of experiments the effects of *trans*-cinnamic acid and L-AOPP were tested on partially purified PAL *in vitro*, and on an *in vitro* translation system, derived from wheat germ. Based on the data from both series of experiments, the possibilities of a specific regulation of PAL activity by *trans*-cinnamic and its hydroxylated derivatives are discussed.

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Materials and Methods

Cell cultures

The cells were propagated as previously described [5]. In order to measure growth of the cell culture packed cell volume (pcv) was determined according to [8].

Determination of soluble proteins

Crude extracts were prepared as already described [5]. The content of soluble proteins in these extracts was determined according to [9].

Extractable PAL activity

PAL was partially purified as already described [5]. The activity was measured as described in [10].

Isolation of polysomes

Cells (10 g fresh weight) were frozen with liquid nitrogen and homogenized in a mortar. After thawing, polysomes were isolated according to [11]. Depending on the purpose, the resulting pellets were washed twice with buffer (10 mM Tris-acetate, 5 mM Mg-acetate, 100 mM KCl, 14 mM 2-mercaptoethanol, 40% glycerol (v/v), 0.5% Triton-X-100, pH 7.6) and resuspended in 500 µl of this buffer or washed twice with medium B [11] and dissolved in 500 µl of this medium. Preparations with a A_{260}:A_{280} ratio of 1.6–1.9 were used for further experiments. Polysomes destined to drive protein synthesis *in vitro* were stored at – 18 °C without loss of activity for 1–2 months.

Separation of polysomes on linear sucrose gradients

150 µl of the suspension in medium B (s.a.) were layered on linear sucrose gradients [11] and centrifuged for 2 h at 131 000 × *g* in a Beckman rotor SW 27.1. The ratio of the monosomes to polysomes was calculated from the peak areas of the profile ($A_{254\,nm}$).

In vitro translation

Polysomes resuspended in buffer were used to drive an *in vitro* system derived from wheat germ according to [12]. *Trans*-cinnamic acid, *p*-coumaric acid, and caffeic acid were applied in concentrations of 10^{-3} M. The incorporation of L-[^{35}S]-methionine into TCA insoluble material was determined.

Results

Effect of trans-cinnamic acid and L-AOPP on growth and soluble protein content of the cells

Cells of *Daucus carota* were grown in the presence of *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid and L-AOPP, each of 10^{-4} M. Growth of the cells was measured by means of packed cell volume (pcv) 9 days after onset of cultivation. At that time maximum anthocyanin accumulation was observed [5]. As shown in Fig. 1 L-AOPP had no effect, whereas all other compounds inhibited growth of the cell suspension, but to a different degree. Among these compounds caffeic acid showed the highest inhibition. If *trans*-cinnamic acid and its hydroxylated derivatives were applied in higher concentrations (10^{-3} M) the cells died within 24 h. These results demonstrate that *trans*-cinnamic acid, *p*-coumaric acid and caffeic have toxic effects on carrot cells grown in suspension culture. On the contrary, L-AOPP has no inhibitory effect on the growth of the cells.

From cells grown in the presence of *trans*-cinnamic acid and L-AOPP (each at 10^{-4} M) soluble proteins were extracted and assayed 3 days after onset of cultivation. In Fig. 2 the values were calculated on the basis of fresh weight and expressed as a percentage of the control. Again, L-AOPP does not affect the protein content whereas *trans*-cinnamic acid lowers the value to 75% of the control. Both compounds administered together are slightly more

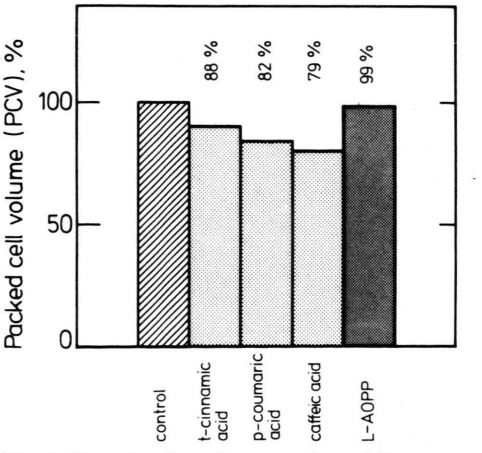

Fig. 1. Growth of a cell suspension of *Daucus carota* in the presence of cinnamic acids (10^{-4} M) and L-α-aminooxy-β-phenylpropionic acid (10^{-4} M). Packed cell volume (pcv) was determined 9 days after onset of cultivation.

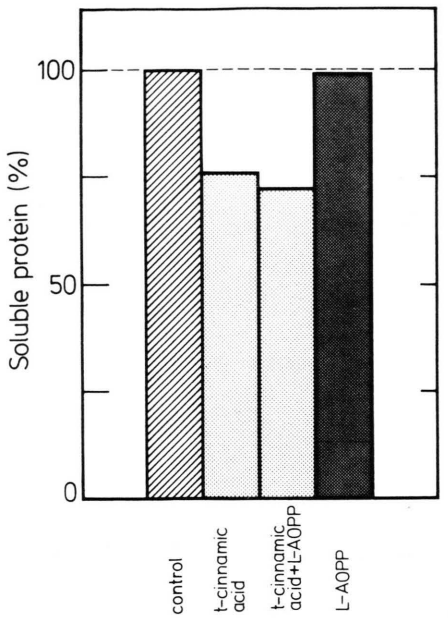

Fig. 2. The content of soluble proteins after treatment of the cell suspension culture of *Daucus carota* with *trans*-cinnamic acid (10^{-4} M) and L-AOPP (10^{-4} M). The protein content of the cells (1 g fresh weight) was determined according to [9] three days after onset of cultivation. The values are expressed as a percent of the control.

inhibitory. In preliminary experiments [5] an increase of all soluble amino acids was observed after treatment of the cells with *trans*-cinnamic acid. This increase of amino acids may be due to a decrease in protein synthesis. Considering all these results, one has to take into account that *trans*-cinnamic acid has inhibitory effects on the protein synthesizing apparatus and consequently growth of the cells is impaired.

Polysomal patterns

In order to obtain an insight into the protein synthesizing capacity *in vivo*, polysomes were extracted 1 day after onset of cultivation. In Fig. 3 such polysomal patterns are shown. After treatment of the cells for 24 h with *trans*-cinnamic acid (10^{-4} M) the ratio now favours the monosomes (Fig. 3b). Normally, a profile is observed which is shown in Fig. 3a. This result clearly demonstrates that the capacity for protein synthesis is reduced after treatment of the cells with *trans*-cinnamic acid. These results are a strong indication of a decrease in protein synthesis *in vivo*.

Inhibition of protein synthesis in vitro

Considering the effects of *trans*-cinnamic acid on growth and protein synthesis *in vivo*, experiments were carried out with an *in vitro* translation system derived from wheat germ. Polysomes, isolated 5 days after onset of cultivation (maximum protein synthesis *in vivo*), were used to drive protein synthesis *in vitro*. In Fig. 4 the ^{35}S-radioactivity incorporated into TCA insoluble material during incubation in the wheat germ system is shown. All cinnamic acids (10^{-3} M) inhibited the translation process. Among the compounds tested in these experiments, caffeic acid was the most detrimental substance, whereas L-AOPP (10^{-3} M), a potent inhibitor of PAL, had absolutely no effect on *in vitro* translation in wheat germ system.

Kinetic data of trans-cinnamic acid and L-AOPP

As mentioned above, *trans*-cinnamic acid is thought to regulate the activity of PAL in a negative feed-back reaction by affecting the amount of extractable enzyme. Therefore the effect of *trans*-cinnamic acid and L-AOPP on the deamination process catalyzed by PAL *in vitro* was tested in order to obtain data on the mode of action of these compounds. In Fig. 5 the substrate saturation curves

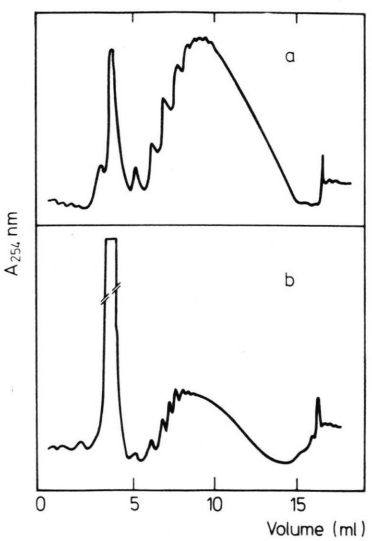

Fig. 3. Polysomal patterns of carrot cells grown in suspension. The cells were harvested one day after onset of cultivation, polysomes were extracted and separated on linear sucrose gradients (125 – 500 mg · ml^{-1}). a) control, b) cells grown in the presence of 10^{-4} M *trans*-cinnamic acid.

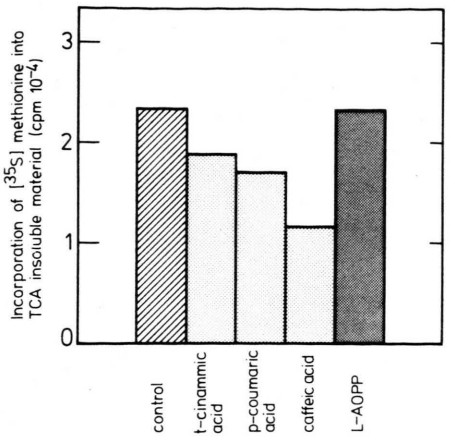

Fig. 4. The effect of cinnamic acids and L-AOPP on *in vitro* translation in a wheat germ system (S-30-fraction) driven by polysomes isolated five days after onset of cultivation from a cell suspension of *Daucus carota*. The S-30-fraction was prepared according to [12]. After 60 min of incubation at 30 °C with L-[^{35}S]methionine the incorporation into TCA insoluble material was determined. Cinnamic acids and L-AOPP were added to the incubation mixture at concentrations of 10^{-3} M.

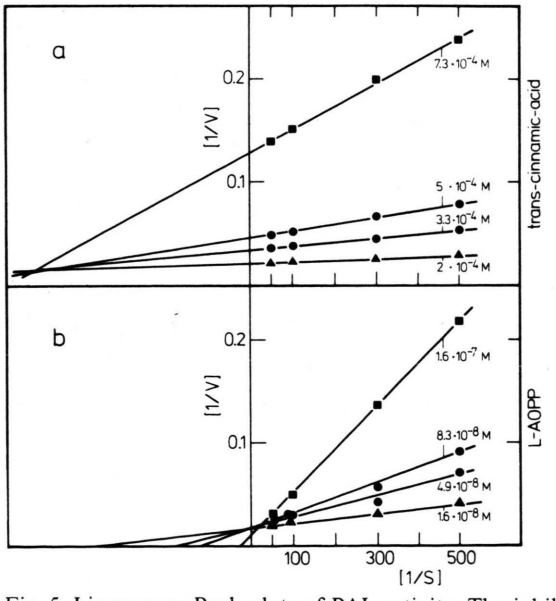

Fig. 5. Lineweaver-Burk plots of PAL activity. The inhibition of the enzyme at various concentrations of a) *trans*-cinnamic acid and b) L-AOPP was tested.

of partially purified PAL [5] were evaluated in a double reciprocal plot according to Lineweaver-Burk. Four concentrations of *trans*-cinnamic acid and L-AOPP were tested for their inhibitory effect on the enzyme *in vitro*. The data clearly demonstrate a *non*competitive inhibition of PAL by *trans*-cinnamic acid (Fig. 5a), despite the findings of several other authors with different plant systems (reviewed in [2]). By comparison, L-AOPP is a competitive inhibitor (Fig. 5b). Very similar results were obtained with methods employed for the evaluation of the kinetic data: the Dixon-, Hanes-, Eadie-Hofstee plots indicate that *trans*-cinammic acid inhibits PAL in a noncompetitive way (data not shown). The K_I was calculated to be 1.8×10^{-4} M, whereas the K_I for L-AOPP was 2.4×10^{-9} M [5]. This high K_I for *trans*-cinnamic acid is also a very strong pointer to a nonspecific inhibition of PAL *in vitro*.

Discussion

Inhibition of enzymes by their end-products is well known in several systems. It is generally accepted that PAL has a regulatory role in the general phenylpropanoid pathway. It is thought that PAL is regulated by *trans*-cinnamic acid, the end-product of the deamination reaction, either in a negative feed-back reaction by direct interaction with the enzyme [2, 4] or by influencing the metabolism of PAL on the level of enzyme synthesis or degradation [1, 13]. Some authors describe a positive feed-back on the synthesis of PAL after application of *trans*-cinnamic acid [3].

In our suspension cultures from anthocyanin-containing carrot *trans*-cinnamic acid had strong toxic effects on protein metabolism and consequently on the growth of these cells, similar to the results found for Jerusalem artichoke tuber tissue [14]. Such effects might not be noticed in systems derived from seedlings, as these are difficult to keep under sterile conditions for periods longer than 48 h.

Growth as well as the content of soluble proteins were affected by *trans*-cinnamic acid. The hydroxylated derivatives of *trans*-cinnamic acid, *p*-coumaric acid and caffeic acid, were more toxic than *trans*-cinnamic acid itself; thus, the majority of experiments were performed using *trans*-cinnamic acid alone.

In preliminary experiments enhanced levels of free amino acids were observed after treatment of the cells with this compound [5]. This might be the result of an interference of *trans*-cinnamic acid with protein synthesis *in vivo*. Polysomal patterns *i.e.*, the ratio of monosomes to polysomes may be used as an indicator for the capacity of protein synthesis *in vivo:* after treatment of the cells with *trans*-cinnamic

acid the polysomes were reduced in favour of the monosomes.

Another line of evidence for an inhibition of protein synthesis came from *in vitro* experiments which support the data obtained *in vivo*. An inhibition of the wheat germ system can be observed at relatively high concentrations of the inhibitory compounds (10^{-3} M). This impairment of the protein synthesizing apparatus is obvious with such short incubation periods of 60 min: these compounds were present in the culture medium for longer periods normally, and under these conditions they develop their detrimental effects at lower concentrations. In preliminary experiments with a heterologous transcription system an inhibition of this process was also recorded (Noé, unpublished). L-AOPP had no effect either on transcription or translation *in vitro*. As demonstrated in a previous publication [5], very high concentrations of *trans*-cinnamic acid were necessary to inhibit PAL *in vitro*. Such concentrations were lethal for our carrot cells. In leaves of higher plants cinnamic acids seem to be glycosylated to avoid poisoning of the cells by these detrimental compounds [15]. Our kinetic data for PAL demonstrate a noncompetitive mechanism of inhibition. By contrast, L-AOPP, an artificial compound, seems to have no side effects *in vivo* as well *in vitro*. From these results it seems to be clear that external application of *trans*-cinnamic acid and its hydroxylated derivatives cannot be involved in a specific regulation of PAL and the general phenylpropanoid pathway.

The results reported here clearly demonstrate, that data on the regulatory role of *trans*-cinnamic acid on the phenylpropanoid pathway have to be interpreted very carefully due to the toxic effects of this compound and its hydroxylated derivatives on protein synthesis and growth of the cells.

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