Induction of chalcone synthase in cell suspension cultures of carrot (\textit{Daucus carota} L. ssp. \textit{sativus}) by ultraviolet light: evidence for two different forms of chalcone synthase

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Abstract. Two cell lines of carrot (\textit{Daucus carota} L. ssp. \textit{sativus}), grown as cell-suspension cultures in the dark, were irradiated with ultraviolet light (315–420 nm) 10 d after the onset of cultivation. Chalcone synthase (CHS) enzyme activity was induced in both cell lines. Anthocyanin synthesis was only stimulated in the anthocyanin-containing cell line DCb. Parallel to the increase in CHS activity there was an increase with time in the amount of one CHS form with an isoelectric point of 6.5 and a molecular weight of 40 kilodaltons (kDa) per subunit. Whereas the anthocyanin-free cell line DCs failed to accumulate anthocyanin, it did stimulate another CHS form with an isoelectric point at pH 5.5 and a molecular weight of 43 kDa per subunit. Both enzyme activities could be separated by isoelectric focusing and stabilized using sodium hydrosulfite as an oxidation protectant. In carrot plants, CHS was restricted to the dark purple petals of the inflorescence (40 kDa) and to the leaves (43 kDa).

Key words: Anthocyanidin – Cell culture (enzyme induction) – Chalcone synthase – \textit{Daucus} – Ultraviolet (enzyme induction)

Introduction

Light, as well as factors like the phytohormone composition of the medium (Ozeki and Komamine 1985) and nutrient depletion, can strongly influence anthocyanin accumulation. The spectral sensitivity of anthocyanin induction varies in different systems, and phytochrome is known to be one of the photoreceptors involved in its photoregulation (Brödenfeldt and Mohr 1986, 1988; Beggs et al. 1987). Regardless of the phytochrome photoreceptor the results of several studies have revealed the participation of photoreceptors specific for ultraviolet (UV) or blue light which often showed a mode of co-operation with phytochrome (Beggs and Wellmann 1985; Oelmüller and Mohr 1985; Take-da et al. 1988).

Experiments dealing with the induction of flavonoid synthesis using cell cultures of parsley yielded similar results (Duell-Pfaff and Wellmann 1982). Studies on the enzymic control after irradiation demonstrated the de-novo synthesis of enzymes involved in both the general phenylpropanoid pathway and the flavonoid pathway (Schröder et al. 1979; Schröder and Schäfer 1980). Both biosynthetic pathways were induced in a coordinated manner (Hahlbrock et al. 1976; Ebel and Hahlbrock 1977).

Special attention was given to the regulation of chalcone synthase (CHS). This enzyme catalyses the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA in order to form naringenin-chalcone (4,2',4',6'-tetrahydroxychalcone; Kreuzaler and Hahlbrock 1975). In cell suspension cultures of parsley, CHS is induced by UV light. The synthesis of enzyme protein is controlled by a transient increase of CHS-mRNA (Kreuzaler et al. 1983). This increase of mRNA was clearly shown to be dependent on the de-novo synthesis of mRNA rather than on the activation of preformed mRNA. Moreover, its synthesis was fluence-dependent (Bruns et al. 1986).

Investigations on CHS genes showed that in some cases there is more than one gene in the haploid genome (Reif et al. 1985; Ryder et al. 1987). Additional evidence for the existence of CHS isoenzymes comes from two purified CHS forms.
from *Spinacia oleracea*. These two types differed in their isoelectric points but had the same molecular weight (Beerhues and Wiermann 1988).

In this report we describe changes in the enzyme activities involved in the flavonoid and general phenylpropanoid pathways after irradiation with UV light. Evidence for two CHS forms with different molecular subunit weights and different isoelectric points is given, and the regulation of these CHS forms is discussed.

Materials and methods

**Chemicals.** [2-14C]Malonyl-CoA was purchased from American Buchler (Braunschweig, FRG). 4-Coumaroyl-CoA was synthesized according to Stöckigt and Zenk (1975). Goat anti-rabbit immunoglobulin G (IgG) (conjugated with alkaline phosphatase), 5-bromo-4-chloro-indolyl phosphate (BCIP), bovine serum albumin (BSA) and malonyl-CoA were products of Sigma (Munich, FRG). Sephadex G25 (fine) was obtained from Pharmacia (Uppsala, Sweden). Ponceau S and Dowex 1 × 2 were purchased from Serva (Heidelberg, FRG).

**Cell cultures.** The experiments were carried out with two cell lines of *Daucus carota* L.ssp. *sativus*. The cell line designated DCb accumulates anthocyanin under dark conditions and consists of anthocyanin-accumulating and non-accumulating cells. Anthocyanin-free parts were selected from the heterogeneous callus and subcultivated on 1% agar medium, resulting in an anthocyanin-free callus culture designated DC (s = selected).

Callus cultures (Seitz and Richter 1970) and cell-suspension cultures (Noé et al. 1980) were propagated as previously described.

**Plant material.** Wild carrots (*Daucus carota* L. ssp. *carota*) were collected near Tübingen (FRG). Only plants with dark-purple petals in the center of the inflorescence were selected. The cultivated carrot (*Daucus carota* L. ssp. *sativus*) was grown in a greenhouse and harvested after 6 months.

**Irradiation.** Dark-grown cell-suspension cultures, cultivated in 250-ml Erlenmeyer flasks on a gyratory shaker (110 rpm, 26° C), were irradiated 10 d after the onset of cultivation with UV light (315–420 nm, \( \lambda_{\text{max}} = 354 \, \text{nm} \), half-band width 40 nm, fluorescence rate 6.3 W·m\(^{-2} \)) from fluorescence tubes (Fit sun 40 W, Wolff System, Freiburg, FRG).

**Protein preparation.** All steps were carried out at 4° C. In the case of suspension cultures, 4 g cells (fresh weight) and 1 g Dowex 1 × 2 equilibrated in 100 mM potassium phosphate buffer (KP), pH 8 were resuspended in 2 ml of 200 mM KP, 50 mM sodium hydrosulfite, pH 7. Homogenization was performed by sonification (30 s, 70 W) using a micro-tip sonifier (Tris), 150 mM glycine, 20% (v/v) methanol.

Frozen plant material was homogenized in a mortar together with liquid nitrogen. One g of cell powder was stirred with 4 ml of 200 mM KP, 50 mM sodium hydrosulfite, pH 7 and 1 g of Dowex 1 × 2 (equilibrated in 100 mM KP, pH 7). After centrifugation (10 min, 27000 g) the supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the CHS assay.

**Chalcone synthase assay.** The activity of CHS was determined according to Hinderer and Seitz (1985). The standard assay mixture contained 150 µl of 100 mM KP, 1.4 mM 2-mercaptoethanol; 1% (w/v) BSA, pH 7.5; 5 µl (1 nmol) of 4-coumaryl-CoA; 5 µl of [2-14C]malonyl-CoA (1.87 nmol, 2.3 KBq); and 50 µl of enzyme extract. The mixture was incubated for 15 min at 30° C. As demonstrated by thin-layer chromatography (TLC) no products other than naringenin were formed if the homogenization buffer contained sodium hydrosulfite.

After IEF separation, CHS activity was determined by cutting the slab gel into small strips and transferring them to 500 µl of the incubation buffer. The assays for enzyme activity were performed as described above but the incubation period was 1 h.

**Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity** was determined according to Kookol and Conn (1961).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.**

Crude protein extracts were subjected to SDS-PAGE according to Laemmli (1970) with a 6% stacking gel and a 10% separation gel. Each slot contained equal amounts of protein. The molecular masses of the polypeptides were determined using the low-molecular-weight calibration kit from Pharmacia.

**Western blotting.** The proteins were transferred electrophoretically to nitrocellulose sheets (pore size 0.45 µm, Schleicher and Schuell, Dassel, FRG) as described by Towbin et al. (1979). This transfer was carried out in the vertical trans-blot cell from Bio-Rad Laboratories (Richmond, Cal., USA) for 12 h (8 V·cm\(^{-1} \)) in 20 mM 2-aminoo-2-(hydroxymethyl)-1,3-propanediol (Tris), 150 mM glycine, 20% (v/v) methanol.

After transfer the blot can be reversibly stained for proteins with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid. The stain can be removed by incubating the membrane in 100 mM Tris-HCl, pH 9.0.

**Immunostaining.** For immunostaining the blot was blocked with 5% (w/v) BSA dissolved in 50 mM Tris, 150 mM NaCl, 0.05% (w/v) Tween 80, and 0.02% (w/v) sodium azide (washing-buffer) for 1 h. After rinsing with washing buffer the blot was incubated in 60 ml of washing buffer containing 2 µl of CHS antiserum developed against CHS from parsley and soybean, respectively (gifts from K. Hahlbrock, Köln, FRG, and R. Welle, Freiburg, FRG, respectively). After an incubation period of about 6 h the sheet has to be washed thoroughly with washing buffer in order to remove unspecifically bound antibodies. Then 5 µl of the second antibody (goat antirabbit IgG conjugated with alkaline phosphatase) directed against the first antibody were dissolved in 60 ml of washing buffer. The second incubation took place for another 6 h. Once again the blot was washed thoroughly and developed in 0.05% (w/v) BCIP, which was presolved in dimethylsulfoxide and adjusted to 14 ml with 1 M Tris-HCl, 1 mM MgCl\(_2\), 0.02% (w/v) sodium azide, pH 8.8. Subunits of CHS appeared as blue bands after several hours. In order to estimate the molecular masses of the subunits, all of the polypeptides on the blot were stained with Ponceau S.

**Immunotitration.** Immunotitration of CHS catalytic activity was performed with crude enzyme preparations from cultured cells that had been exposed to UV light for 48 h ten days after the onset of cultivation. Chalcone synthase (about 0.3 pkat)