Phytochemistry, 1979, Vol. 18, pp. 581–584. Pergamon Press. Printed in England

LIGHT INDUCED CHANGES IN ANTHOCYANIN CONCENTRATION, ACTIVITY OF PHENYLALANINE AMMONIA-LYASE AND FLAVANONE SYNTHASE AND SOME OF THEIR PROPERTIES IN *BRASSICA OLERACEA*

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(*Received* 29 *June* 1978)

Key Word Index—*Brassica oleracea* cv Red Danish; Cruciferae; anthocyanins; biosynthesis; enzymes; PAL; flavanone synthase.

Abstract—Seedlings of red cabbage, *Brassica oleracea* cv Red Danish, germinated in the dark, rapidly produced anthocyanins upon illumination. The anthocyanin production increased up to six days of illumination time. The activity of phenylalanine ammonia-lyase increased rapidly in illuminated seedlings to a maximum at 8 hr and declined thereafter to dark levels. During this period the activity of flavanone synthase, the first enzyme responsible for the establishment of C_{15} flavonoid skeleton, paralleled that of the anthocyanin concentration. The crude flavanone synthase has a pH optimum at around 8, a molecular weight of *ca* 120000, and is able to utilize only *p*-coumaryl-CoA as co-substrate for the production of flavonoids.

INTRODUCTION

The physiology of anthocyanin synthesis has been more extensively examined than the synthesis of any other type of phenolic. Thus it was known relatively early [1] that the synthesis of anthocyanins in plants is under a vigorous photocontrol and that this reaction is governed by a red/far red photoreceptor system. Red cabbage seedlings have been extensively used because of their short period of germination and the production of anthocyanins in relatively large amounts upon illumination. The first major attempts at the elucidation of the biosynthetic path of anthocyanins in red cabbage seedlings were carried out by Frey-Wyssling and Blank [2] who correlated the metabolism of sugars and nitrogen with the development of anthocyanins.

Progress in the last decade in the descriptive enzymology of flavonoid synthesis in parsley seedlings and cell suspension cultures (for a comprehensive review see [3]) made possible the establishment of the complete biosynthetic reaction sequence for malonylgraveobiosid B [4]. While the flavone and flavonol glucoside pathway is reasonably well established, only few coordinated data are available on the biosynthetic reactions involved in the formation of anthocyanins [5, 6].

This report describes the correlation of anthocyanin production with phenylalanine ammonia-lyase (PAL) and with flavanone synthase, the first enzyme responsible for the establishment of the C_{15} skeleton of flavonoid compounds as well as some of the properties of this enzyme. Cabbage has been chosen as plant material for this investigation because of the relatively large amount of data on its anthocyanin production. The elucidation of the structure of the anthocyanins present in red cabbage seedlings [7] makes this plant ideally suited for this investigation.

RESULTS

Changes in seedlings through germination and illumination

Red cabbage seedlings developed rapidly after imbibition and synthesized anthocyanins upon germination in the dark. Their anthocyanin content was greatly increased by exposure to light even at early stages of development (Table 1). While the fresh weight increased rapidly, the protein content declined during development. The PAL activity increased in imbibed seeds within 48 hr to a level which remained constant in the dark. The experiments reported here were initiated *ca* 72 hr after imbibi-

Table 1. The development of red cabbage seedlings in darkness and their ability to synthesize anthocyanins upon illumination

Treatment from imbibition	Weight mg/sdl	Protein mg/sdl	PAL pkat/sdl	Anthocyanin nmol/sdl
None, seedcoats removed	4.7	0.48	0.1667	0
24 hr darkness	8.0	0.43	0.2834	0.32
48 hr darkness	14.1	0.42	1.016	0.46
24 hr dark + 24 hr light				8.66
72 hr darkness	28.0	0.33	2.8501	5.13
48 hr dark + 24 hr light				28.40
96 hr darkness	34.9	0.35	1.066	6.70
72 hr dark + 24 hr light				22.00

Table 2. The distribution of anthocyanin, protein and PAL in dark-grown red cabbage seedlings given 15 hr light

Part	Anthocyanin nmol per sdl	Anthocyanin % of total	Protein mg/sdl	Protein % of total	PAL pkat/sdl	PAL % of total	Spec. act. PAL (μkat/kg protein)
Cotyledon	13.2	64	0.176	72	1.167	47	6.7
Top 1/3 of hypocotyl	4.8	23	0.025	10	0.583	23	22.8
Mid 1/3 of hypocotyl	1.7	8	0.023	9	0.450	18	19.3
Bottom 1/3 of hypocotyl	0.9	4	0.021	9	0.300	12	14.3
Total hypocotyl	7.4	36	0.069	28	1.336	53	19.3

tion. Table 2 shows the distribution of anthocyanin, protein and PAL in the seedlings after a 15 hr light treatment. Although most of the anthocyanin was found in the cotyledons, the highest specific activity of PAL was in the hook region of the hypocotyls.

The dark-grown seedlings therefore contain small, but significant amounts of anthocyanin before illumination (0.6 μmol/g) (Fig. 1). The amount of pigment increases in the dark for another 24 hr and shows a continuous decline thereafter for the next two days, when it reaches a plateau at *ca* 0.35 μmol/g tissue concentration. Upon illumination of the seedlings with continuous light, the anthocyanin content increased rapidly and almost linearly in the first 60 hr. After this illumination period the rate of pigment production progressively decreased up to 192 hr illumination time, the longest duration of the experiments (Fig. 1). The highest amount of anthocyanin produced during these periods was 5 μmol/g seedlings, 8-fold the amount produced in the dark.

Cotyledons were clearly the main site of anthocyanin biosynthesis with the maximal pigment concentration of 6.5 μmol/g tissue (Fig. 1b); hypocotyls contained considerably lower amounts (4.2 μmol/g tissue) after 192 hr illumination (Fig. 1c).

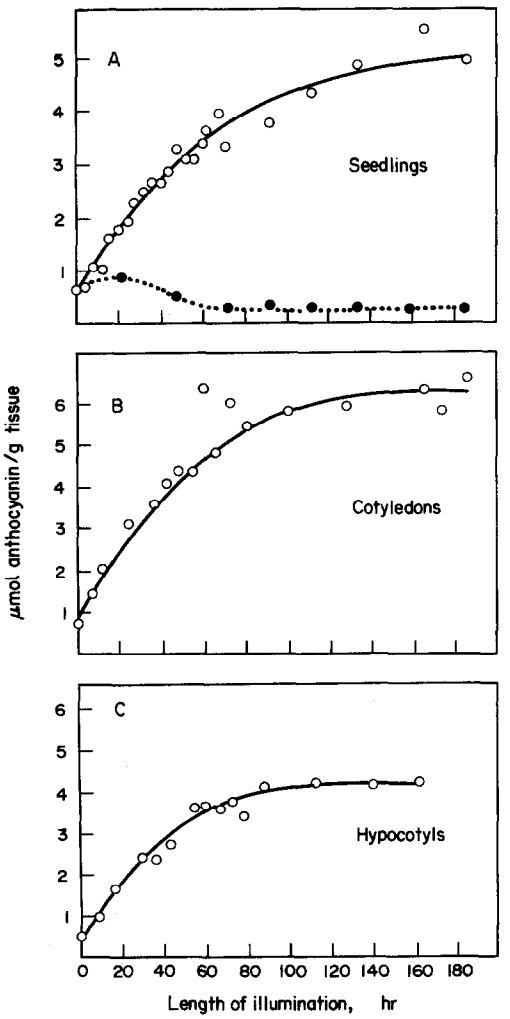

Fig. 1. Changes in the anthocyanin concentration in dark germinated red cabbage seedlings upon illumination. A: Seedlings; B: cotyledons; C: hypocotyls. Seedlings were grown in the dark. Illumination started three days after imbibition. The dotted line represents anthocyanin formation in dark.

Changes in the activity of phenylalanine ammonia-lyase and flavanone synthase upon illumination

The PAL activity of illuminated seedlings increased rapidly after illumination as shown in Fig. 2. After 8 hr the activity decreased and fell to the level characteristic of seedlings maintained in the dark. The increase in the activity of flavanone synthase followed a different pattern and increased steadily throughout the duration of illumination (Fig. 3a). As is the case with the production of anthocyanins, the largest amount of activity was found in the cotyledons (Fig. 3b), although the pattern of the change in activity was the same in both cotyledons and hypocotyls (Fig. 3c). Hypocotyls, in spite of showing high specific activity for the enzyme, did not contain sufficient protein for practical purposes.

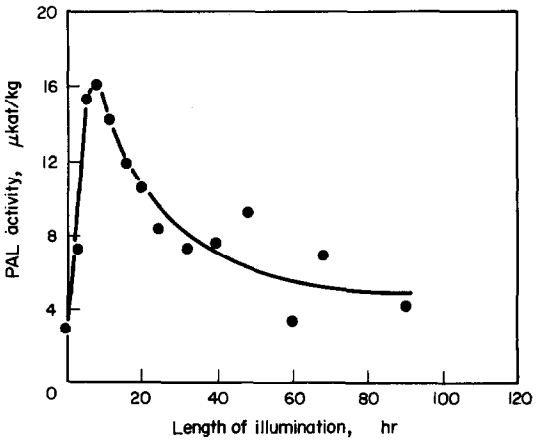

Fig. 2. Changes in the activity of phenylalanine ammonia-lyase in dark germinated red cabbage seedlings upon illumination.

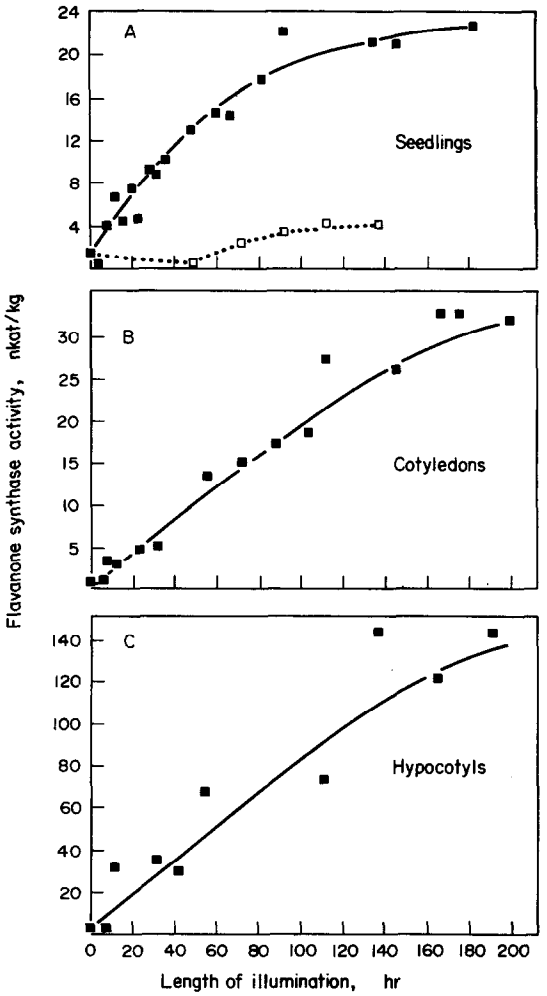

Fig. 3. Changes in the activity of flavanone synthase in dark germinated red cabbage seedlings upon illumination. A: Seedlings; B: cotyledons; C: hypocotyls. Seedlings were grown in the dark. Illumination started three days after imbibition. Dotted line represents activity of flavanone synthase in dark.

Properties of the flavanone synthase

The activity of flavanone synthase was investigated in the pH range 3–10 using buffer systems C, D and E. The enzyme had its maximum activity around pH 8, similar to the parsley flavanone synthase [8] and showed *ca* 20% higher activity in 200 mM phosphate buffer (C) than in McIlvain buffer (D) of similar ionic strength. The activity of the enzyme in glycine–NaOH buffer (E) in the pH 8–8.3 region was approximately 40% of that found in phosphate buffer.

The molecular weight of the flavanone synthase was determined to be 120000 on a Ultrogel AcA 44 column (see Experimental). The substrate specificity of the flavanone synthase was investigated in the pH range of 3–10 with cinnamyl-, *p*-coumaryl-, caffeyl-, ferulyl-, 5-hydroxyferulyl and sinapyl-CoA as substrates in the presence of ascorbic acid in the incubation mixtures. Only *p*-coumaryl-CoA was utilized as substrate. Under the conditions of the experiment, no early release products of the flavanone synthase reaction [9] such as bisnoryangonin, *p*-hydroxybenzalacetone and 4-hydroxy-5-6-dihydro-6-(4-hydroxyphenyl)-pyrone could be detected.

DISCUSSION

Seeds of red cabbage *Brassica oleracea* cv Red Danish do not contain any measurable amount of anthocyanins [2]. Upon germination in the dark, however, a small but significant amount (0.6 μmol/g seedling) of these pigments is formed. Since the synthesis of anthocyanins in red cabbage seedlings has been shown to be under strict photocontrol [1], another mechanism not involving any photoeffects has to be responsible for this early synthesis of pigments. It has been suggested [10] that β-oxidation of fatty acids and the production of C_2 or C_3 fragments could initiate this photo-independent synthesis of anthocyanins. The production of pigments in the dark declines after 48 hr of germination (Fig. 1a; the first point in the figure, 0 hr illumination time, was taken three days after imbibition), and reached a steady level of *ca* 0.3 μmol/g seedling for the remaining time of the experiment. When the seedlings were illuminated a rapid production of anthocyanin pigments was observed. The pigment production remained approximately linear for the first 60 hr of illumination but the rate of synthesis declined for the remainder of the experiment. The same pattern of anthocyanin synthesis was observed also in the individual organs. Cotyledons (Fig. 1b) had a much higher rate of synthesis than did hypocotyls (Fig. 1c). Phenylalanine ammonia-lyase showed only ephemeral changes in activity upon illumination. The increase in activity of flavanone synthase, the first enzyme responsible for the establishment of the C_{15} flavonoid skeleton, paralleled the formation of anthocyanins in the seedling as well as in cotyledons and hypocotyls (Fig. 3). In Fig. 3 the increase in the specific activity of this enzyme is shown. Cotyledons (Fig. 3b) showed the highest protein concentration and enzyme activity during illumination. Hypocotyls (Fig. 3c), in spite of showing an extremely high specific activity of flavanone synthase, contained insufficient amounts of protein for practical work.

The properties of the flavanone synthase from the red cabbage seedlings so far investigated are similar to those described for the same enzyme found in cell suspension cultures of parsley [11, 12]. The enzyme has its pH optimum at around pH 8 and showed the highest activity in 200 mM phosphate buffer. In its MW, it closely resembles that of the parsley enzyme. The substrate specificity of the flavanone synthase was first established in parsley cell suspension cultures [9] in the presence of 2-mercaptoethanol. Under the conditions described in that report, the parsley flavanone synthase successfully utilized *p*-coumaryl-CoA for the synthesis of naringenin, while from caffeyl- and ferulyl-CoA's mainly short chain condensation products were released prematurely. A recent report on flavanone synthase from tulip anthers [13] described the utilization of caffeyl-CoA also as a true substrate toward the synthesis of the flavanone eriodictyol in the presence of ascorbate. Because of the possibility that the flavanone synthase behaves differently in the presence of ascorbate than with 2-mercaptoethanol, our experiments were carried out with either 2-mercaptoethanol or ascorbate. Since there was also the possibility that the different substrates are utilized by the same enzyme at different pH values, all the experiments were carried out in the entire pH range of 3–10. Under these circumstances we found that only *p*-coumaryl-CoA acted as substrate. While in the presence of 2-mercaptoethanol in the incubation mixture some incomplete 'short chain

products' were released when caffeyl-, feruloyl and sinapyl-CoA's were used at around pH 8, no incorporation of these compounds, or others mentioned in the Experimental, could be observed in the presence of ascorbate in any of the pH ranges investigated. Therefore it is clear that only *p*-coumaryl-CoA is utilized as co-substrate in the biosynthesis of flavonoids in red cabbage, and that naringenin is the first flavanoid formed. A report by Saleh *et al.* [14] appeared recently showing the utilization of caffeyl-CoA as a substrate for the flavanone synthase from *Happlopappus* at pH's differing from the optimal utilization of *p*-coumaryl-CoA. A similar mechanism in red cabbage seedlings, however, could not be substantiated by us.

EXPERIMENTAL

Plant material and growth conditions. Red cabbage (*Brassica oleracea* cv Red Danish) seeds were obtained from Seedway, Inc., Hall N.Y. Germination rate 90%. The seeds were soaked overnight in H_2O and germinated on two layers of moist Whatman No. 1 filter paper in clear plastic boxes (27 × 19 × 10 cm) in the dark in a thermostated growth chamber at 20°. The seedlings were illuminated continuously thereupon (200 μEinstein m^{-2}. sec^{-1}) with an equal number of Cool White and Grow Lux fluorescent lights and samples taken at intervals up to 192 hr illumination time.

Materials. Cinnamyl-, *p*-coumaryl-, caffeyl, feruloyl-, 5-hydroxyferulyl and sinapyl-CoA's were synthesized as described previously [9]. [2-^{14}C]-Malonyl-CoA was obtained from New England Nuclear, Boston, MA; Ultrogel AcA 44 from LKB Instruments; aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A from Pharmacia. Naringenin was obtained from Sigma, eriodictyol and homoeriodictyol were from our laboratory collection.

Buffers. (A) 200 mM K_2HPO_4–H_3PO_4 pH 8.0 containing 5 mM mercaptoethanol was used for the preparation of the crude protein extracts. (B) 10 mM K_2HPO_4–H_3PO_4 buffer, pH 8.0, containing 3 mM mercaptoethanol was used as solvent for the crude enzyme at the establishment of pH optima. The optimum activity of the enzyme was determined in the pH range 3–10 using 200 mM McIlvain (C); 200 mM K_2HPO_4–H_3PO_4 (D) and 100 mM glycine–NaOH (E) buffer, all containing 5 mM ascorbic acid. All expts were carried out in duplicate and data presented here were obtained from three separate series of experiments.

Determination of anthocyanin concentration. 100–300 mg seedlings, cotyledons or hypocotyls were extracted with 5 ml MeOH (0.1% HCl) for 10 min at room temp., the extract filtered through a nylon gauze and the extraction repeated twice with 2 ml MeOH (0.1% HCl). The methanolic extract was made up to 10 ml in a volumetric flask and the anthocyanin concentration determined as described previously [11].

Crude protein extracts. Extraction procedure I. Approximately 100–200 mg seedlings, cotyledons or hypocotyls were homogenized in the presence of 50 mg granular silica, 100 mg PVP and 1.0 ml buffer A in chilled 1.5 ml Eppendorf centrifuge tubes with a conically shaped rotating (100 rpm) glass rod for 5 min. The homogenate was centrifuged thereafter in the same tubes for 2 min at 12500 *g*. The supernatant was stirred with 100 mg Dowex 1 × 2 (PO_4 form, pH 8.0) for 5 min and the ion exchange resin was removed by centrifugation at 12500 *g* for 1 min in an Eppendorf microcentrifuge. The supernatant was added to a Pharmacia PD-2 column and chromatographed with 3 ml buffer A. The eluate, free of phenolics and other low MW compounds was used for the determination of flavanone synthase activity. *Extraction procedure* II. 20 g seven-day-old seedlings grown under continuous illumination were homogenized with 3 g granular silica, 5 g PVP and 25 ml buffer A. The homogenate was centrifuged at 18000 *g* for 20 min and the supernatant stirred with 5 g Dowex 1 × 2 (PO_4 form, pH 8.0) for 30 min. After removal of the ion exchange resin, the protein solution was rebuffered on a Sephadex G-25 column (2.5 × 40 cm) using buffer B. The elution was monitored at 280 nm, the protein peak collected and used for the determination of the pH optimum.

Determination of enzyme activities. PAL was assayed as previously described [15]. Flavanone synthase: 100 μl samples were incubated with 5 μl *p*-coumaryl-CoA (1 nmol) and 5 μl [2-^{14}C]-malonyl-CoA (1.5 nmol, 1.1 × 10^5 dpm) for 20 min at 30°. The ^{14}C naringenin was separated and counted as described in [8]. Incubations for the determination of the pH optimum were carried out with 100 μl crude enzyme preparation in buffer B and 100 μl of the corresponding buffer under conditions identical to those described above.

In incubation mixtures for the determination of the substrate specificity of the crude flavanone synthase preparation, 1 nmol of the corresponding co-substrate was used instead of *p*-coumaryl-CoA. The chromatograms from these incubations were developed with authentic reference compounds as carriers and were analysed both in a Packard Model 72000 Radiochromatogram Scanner and by liquid scintillation spectrometry.

Determination of the molecular weight of the flavanone synthase. The MW of the flavanone synthase was determined on a Ultrogel AcA 44 column (2.5 × 85 cm) using buffer A as eluent at the flow rate of 5.63 cm/hr. The column was calibrated with aldolase (158000), ovalbumin (45000), chymotrypsinogen A (25000) and ribonuclease A (13700).

Protein determination. In the PAL experiments protein was measured by the biuret method [16]. In the flavanone synthase experiments, the method of ref. [17] was used.

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