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ANTHOCYANIN COMPOSITION OF *SINAPIS ALBA*, LIGHT INDUCTION OF ENZYMES AND BIOSYNTHESIS

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Key Word Index—*Sinapis alba*, Cruciferae, anthocyanins, acyl esters, cyanidin 3-sambubioside-5-glucoside, cyanidin 3-sambubioside-5-sophoroside, (2*S*)flavanone 3-hydroxylase; (+)-dihydroflavanol 4-reductase

Abstract—The two major pigments of light-grown mustard (*Sinapis alba*) seedlings were identified as cyanidin 3-sambubioside 5-glucoside and cyanidin 3-sambubioside 5-sophoroside esterified with malonic, *p*-coumaric, ferulic, and sinapic acids The activities of the enzymes (2*S*)-flavanone 3-hydroxylase and (+) dihydroflavanol 4-reductase and anthocyanin accumulation were determined in mustard seedlings at various times after illumination with red light (λ_{max}= 660 nm). Both enzymes showed a drastic increase in activity after illumination and reached their maxima at the time of rapid anthocyanin accumulation. The results are consistent with the involvement of these enzymes in anthocyanin biosynthesis.

INTRODUCTION

The induction of anthocyanin synthesis in mustard (*Sinapis alba*) seedlings by light has been used extensively to study the role of sensor pigments (phytochrome, cryptochrome, UV-B photo receptor) [1, 2]. However, the nature of the anthocyanins present in mustard has not been clarified It was reported that five anthocyanins could be separated by TLC [3]. The aglycone of all five pigments was cyanidin and four of the pigments seemed to be esterified with sinapic acid. The nature of the glycosidic residues remained unknown.

Studies on the biosynthesis of anthocyanin in mustard cotyledons demonstrated phytochrome-mediated increases in the activities of phenylalanine ammonia lyase [4, 5] and chalcone synthase [5] which are due to synthesis *de novo* of enzyme protein. The activity of chalcone isomerase also increased in mustard cotyledons under continuous far-red light [6].

Recently it has been shown that flavan-3,4-*cis*-diols (leucoanthocyanidins) are intermediates in anthocyanin biosynthesis [7] The enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols in flower extracts of *Matthiola incana* was correlated with anthocyanin-positive genotypes [8]. The enzyme preceding this step, (2*S*)-flavanone 3-hydroxylase, has also been characterized [9].

We now report partial structures for the major anthocyanins of mustard and on the red light-induced activity changes of flavanone 3-hydroxylase (F3H) and (+) dihydroflavonol 4-reductase (D4R)

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Abbreviations (2*S*)-Flavanone 3-hydroxylase, F3H, (+) dihydroflavonol 4-reductase, D4R

RESULTS AND DISCUSSION

By PC, two major pigments were isolated from extracts of fresh mustard seedlings grown under white light The pigments were further purified by HPLC. After saponification with 2 N NaOH the pigments were again purified by PC and HPLC.

Saponified anthocyanin 1 (sap 1) gave on acid hydrolysis cyanidin, glucose and xylose By partial hydrolysis it yielded cyanidin 3-glucoside and 5-glucoside, cyanidin 3,5-diglucoside and a trace of cyanidin 3-sambubioside. Hydrogen peroxide oxidation yielded sambubiose and glucose. FABMS of sap 1 gave $[M]^+$ 743 ($C_{32}H_{39}O_{20}$ requires 743) with fragments of 581 $[M-162]^+$ corresponding to loss of glucosyl, 449 $[M-294]^+$ indicating loss of xylosyl and glucosyl, and 287 corresponding to cyanidin Absorption maxima of sap 1 in 0.1% MeOH–HCl were 279 and 527 nm, and E_{440}/E_{max} [5] was 10.5. Sap 1 was thus identified as cyanidin 3-sambubioside 5-glucoside

Saponified anthocyanin 2 (sap 2) gave by acid hydrolysis cyanidin, glucose and xylose. On partial acid hydrolysis it yielded cyanidin 3-glucoside and 5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside (trace) and two other unidentified glycosides (compound A and B). To clarify the structure of the intermediates A and B both compounds were purified by preparative TLC of the partial hydrolysates with solvent 3. By partial hydrolysis compound A gave only cyanidin 5-glucoside while compound B yielded cyanidin 3-glucoside and 5-glucoside, cyanidin 3,5-diglucoside, compound A and cyanidin. The results showed that the compound A was cyanidin 5-diglucoside and B was cyanidin 3-glucoside 5-diglucoside Accordingly the above results suggest sap 2 is a tetraglycoside of cyanidin.

Furthermore, hydrogen peroxide oxidation of sap 2 pigment gave sambubiose and sophorose, which were

identified by descending paper chromatography using solvents 6–8. In addition, FABMS gave $[M]^+$ 905 ($C_{38}H_{49}O_{25}$ requires 905), with fragments 611 $[M-294]^+$ indicating a loss of xylosyl and glucosyl, 581 $[M-324]^+$ corresponding to loss of 2 glucosyl, and 287 (cyanidin) Absorption maxima in 0 1% MeOH–HCl were 279 and 527 nm, and E_{440}/E_{max}(%) was 12.6. From these results, sap 2 is established as cyanidin 3-sambubioside 5-sophoroside

Sinapis anthocyanin 1 *and* 2

By complete acid hydrolysis both pigments gave cyanidin, glucose and xylose On paper electrophoresis for 1 5 hr both pigments moved towards the anode, both +6 mm on Toyo 51B filter paper in 0 1 M acetate buffer pH 4 4 at 40 V/cm and 0.9 mA/cm, suggesting that the pigments were zwitterionic [10] Absorption maxima of the pigments in 0.1% MeOH–HCl were 283, 299, 325 and 532 nm Values of $E_{UV-max}/E_{vis-max}$ were 1 07 (No 1) and 0 88 (No. 2) respectively, suggesting the presence of phenolic acids in the pigments After saponification malonic acid was identified by TLC [11] In addition, sinapic, *p*-coumaric and ferulic acid were detected using benzene–2N NaOH (1 1, upper phase), water and BAW as solvents Both pigments No 1 and No 2 gave one spot on TLC and one peak on HPLC However, FABMS showed both pigments to be mixtures $[M]^+$ of No 1· 1181 and 1211, and No. 2· 1343 and 1373 respectively The results strongly suggest that pigment 1 is a mixture of cyanidin 3-sambubioside 5-glucoside acylated with malonic, sinapic and *p*-coumaric acid ($C_{55}H_{57}O_{29}$ requires 1181) and the same glycoside acylated with malonic, sinapic and ferulic acid ($C_{56}H_{59}O_{30}$ requires 1211) and that pigment 2 is a mixture of cyanidin 3-sambubioside 5-sophoroside acylated with malonic, sinapic and *p*-coumaric acid ($C_{61}H_{67}O_{34}$ requires 1343) and the same glycoside acylated with malonic, sinapic and ferulic acid ($C_{62}H_{69}O_{35}$ requires 1373)

The anthocyanin composition of mustard is therefore similar to that of other Cruciferae Both *Raphanus sativus* [12] and *Brassica oleracea* [13, 14] contain various acylated derivatives of pelargonidin and cyanidin 3-sophoroside-5-glucosides

Induction of enzymes by light

Beggs *et al* have shown that if dark grown mustard seedlings are illuminated with far-red light (740 nm) anthocyanins and quercetin are formed, whereas by illumination with red light (660 nm) only the synthesis of anthocyanin is induced [6] After illumination of mustard seedlings with red light the activity changes of F3H and D4R and accumulation of anthocyanins were determined (Fig 1). As illustrated in the figure the maximum activity of D4R was reached at 12 hr after onset of illuminations, whereas F3H had a rather broad maximum In the dark-grown seedlings activities of these enzymes were very low which is consistent with the fact that dark-grown seedlings do not contain anthocyanins Maximal enzyme activities were reached at the time of rapid anthocyanin accumulation However, a correlation of enzyme activities with anthocyanin accumulation should take into account that anthocyanin synthesis is almost completely confined to the lower epidermis of mustard cotyledons [6] For a correlative analysis only enzyme activities of the lower epidermis should therefore be used In our case not enough tissue was available for such an analysis

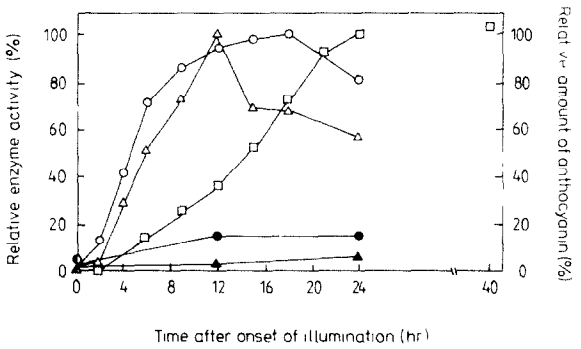

Fig 1 Changes in anthocyanin accumulation and enzyme activities after illumination of dark-grown mustard seedlings with red light 100% represents, for anthocyanin (□ – –□), an A_{533} of 0 59 per seedling Ten seedlings were used per extraction 100% represents, for D4R (△ – –△), 2 9 fkat and for F3H (○ — ○), 40 fkat per seedling Fifty seedlings were used for extraction Filled out symbols are dark controls

EXPERIMENTAL

Radioactive substrates for the enzyme assays, anthocyanins, phenolic acids and disaccharides were from our laboratory collection *Sinapis alba* seeds were a gift form Dr E Wellmann, Freiburg Sambubiose was prepared from cyanidin 3-sambubioside by H_2O_2 oxidation Cellulose plates (plastic sheets, Merck) were used for TLC and Toyo 51B filter paper for PC, with the following solvents 1, HOAc–HCl–H_2O (30 3 10), 2, HCOOH–HCl–H_2O (5 2 3) for anthocyanidins, 3, HOAc–HCl–H_2O (15 3 82), 4, *n*-BuOH–2N HCl (1 1, upper phase), 5, *n*-BuOH–HOAc–H_2O (4 1 5, upper phase), 6, 1% HCl for anthocyanins, 7, *n*-BuOH–toluene–pyridine–H_2O (5 1 3 3), 8, *n*-BuOH–EtOH–H_2O (4 1 2 2), and 9, *n*-BuOH–pyridine–H_2O (6 3 1) for sugars Acid hydrolysis, saponification and H_2O_2 oxidation were carried out as described previously [11] FABMS spectra were obtained with a JEOL-HX100 tandem spectrometer and xenon as the source of the fast atom beam (6 keV) The mass spectrometer was operated at 5 kV accelerating voltage

Isolation of anthocyanins for structural determination Seedlings were grown on wet filter paper under continuous white light or in the greenhouse Pigments were extracted from fresh 5-day-old seedlings with MeOH–HOAc–H_2O (10 1 9, MAW) and the extract evapd to dryness *in vacuo* at 30° The residue was dissolved in MAW and was passed through a Sephadex column (2 5 × 23 cm) in the same solvent The pigment fraction was evapd to dryness and further purified by mass paper chromatography with solvent 5, whereby two major pigment bands (Nos 1, 2) were obtained. Each pigment was purified by mass paper chromatography with 15% HOAc Finally, both pigments were purified by HPLC on an ODS (10–20 μm) column (7 × 300 mm) using HCOOH–MeOH–H_2O (4 15 31) as a solvent

Purification of saponified anthocyanins Anthocyanins 1 and 2 purified as above were saponified respectively by treatment with 2 N NaOH for 45 min under N_2 at room temp After acidification with 2 N HCl, each reaction mixture was passed through

an Amberlite XAD-7 column (1.2 × 20 cm). The column was washed with H_2O and the pigment was eluted with MAW. Both saponified pigments (sap 1 and sap 2) were purified by mass paper chromatography with solvent 5 and by HPLC (ODS 10–20 μm, 7 × 300 mm) using HCOOH–CH_3CN–H_2O (2:2 21) as a solvent. Each pigment showed only one spot on TLC in 4 solvents.

Irradiation of seedlings. Seedlings were germinated on wet filter paper for 36 hr in the dark. The seedlings were then irradiated with red light (λ_{max} = 660 nm, halfbandwidth = 18 nm, fluence rate = 0.67 W/m^2). Anthocyanins were extracted and measured as described [15].

Preparation of enzyme extracts. For dihydroflavonol 4-reductase: 50 mustard seedlings (0.5 g fr. wt) were ground in a mortar with 0.25 g Dowex 1 × 2, 0.1 g quartz sand and 1 ml of 0.1 M Tris–HCl (pH 7.5) containing 10% glycerol and 20 mM Na-ascorbate. The slurry was centrifuged for 5 min at 10000 rpm in an Eppendorf centrifuge. For buffer change the supernatant was filtrated through a Sephadex G-25 column (1 ml) which had been equilibrated with 0.2 M K-Pi (pH 6.8) containing 20 mM Na-ascorbate.

For assay of flavanone 3-hydroxylase the extract without change of buffer was added to the assay mixture.

Enzyme assays. The enzyme assays have been described previously flavanon 3-hydroxylase [9], dihydroflavonol 4-reductase [8].

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