Phytochemistry, Vol. 20, No. 6, pp. 1259–1263, 1981.
Printed in Great Britain.

0031-9422/81/061259-05 $02.00/0

THE EFFECT OF BLUE LIGHT ON FREE AND ESTERIFIED PHENOLIC ACIDS IN ETIOLATED GHERKIN TISSUES

E. ELLEN BILLETT*, RENÉE J. GRAYER-BARKMEIJER, C. B. JOHNSON and J. B. HARBORNE

Department of Botany, The University, Whiteknights, Reading, RG6 2AS, Berkshire, U.K.

(*Received* 29 *October* 1980)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; gherkin; cinnamic acid; *p*-coumaric acid; sugar esters; HPLC.

Abstract—The major phenolic acid found in gherkin tissues is *p*-coumaric acid, although cinnamic and caffeic acids are also present; these occur both free and esterified with glucose. Following blue light treatment the concentration of cinnamic acid relative to *p*-coumaric acid in the pool of free acids is reduced, but the total concentration of free phenolic acids increases. Blue light treatment results in a five-fold increase in the concentration of phenolic acid esters between 2 hr and 6 hr and the ester pool subsequently declines. These results are discussed in relation to the known changes in phenylalanine ammonia-lyase (PAL) activity in those tissues; it is concluded that an increase in PAL activity is an essential prerequisite for the increase in phenolic esters but that the increased enzyme activity is not due to release from repression by free phenolic acids.

INTRODUCTION

The extractable activities of several enzymes of the phenylpropanoid pathway, in particular phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and cinnamic acid 4-hydroxylase (CA4Hase, EC 1.14.13.11), are altered by various stimuli including light, excision, cold stress and infection [1]. It has also been shown that changes in the extractable activities of PAL and CA4Hase can be mediated by manipulating the endogenous pools of cinnamic acid and *p*-coumaric acid [2–4]. However, no attempt has been made to measure changes in the absolute concentrations of free phenolic acids in plant tissues in response to external stimuli to determine whether these acids could be involved in regulating enzyme levels and activity *in vivo*.

When etiolated gherkin tissues are exposed to blue light there is a rapid increase in the concentration of sugar esters of phenolic acids, the main phenolic products in these tissues [5]. This increase is associated with a transient increase in the extractable activity of PAL in both cotyledons and hypocotyls, reaching a peak at about 3.5 hr and then declining, whilst in cotyledons concurrent changes in CA4Hase activity also occur [4]. Experiments using exogenously applied cinnamic acid have suggested that whilst cinnamic acid has the potential to reduce the extractable activity of PAL in these tissues, it cannot mediate changes in the extractable activity of the CA4Hase [4]. An estimate of the relative concentrations of free and esterified acids is important since the endogenous pool would be expected to have a greater role in controlling enzyme levels, being less likely to be sequestered in storage compartments. The present paper reports the effects of blue light on the concentration of (a) free phenolic acids and (b) phenolic acids esterified with sugars, in gherkin tissues using HPLC, TLC and PC.

RESULTS

A preliminary analysis of methanolic extracts by two-dimensional (2D) PC and TLC on fluorescent plates showed that cotyledons contained flavonoids and a number of other phenolic compounds in addition to free and bound phenylpropanoid acids. The phenolic fraction of the hypocotyl extracts, on the other hand, was composed mainly of phenylpropanoid acids and their esters. HPLC analysis was therefore restricted to hypocotyl tissue but both cotyledon and hypocotyl tissues were also analysed by 2D PC and by TLC.

Identification of free and esterified cinnamic acids in etiolated and blue light-treated gherkin tissues by PC and TLC

Using 2D PC it was shown that dark-grown gherkin cotyledons and hypocotyls contained small amounts of a phenolic acid ester (ester 1, Table 1) which appeared blue or colourless under long wave (360 nm) UV light, becoming purple when exposed to NH_3 gas. Following acid hydrolysis and analysis by one-dimensional (1D) TLC in BzAW† and 1D PC in BAW and CAW, the phenolic component of this ester was shown to be *p*-coumaric acid, having identical R_fs to a *p*-coumaric acid marker (see Table 2). Dark-grown tissues also contain very small amounts of two other esters (esters 2 and 3, Table 1) which, following acid hydrolysis and analysis by TLC on fluorescent plates, were shown to be esters of ferulic and caffeic acids. The hydrolysis products were impossible to detect by PC. The concentration of all esters increased following exposure to blue light.

* Present address: Department of Biochemistry, University Hospital and Medical School, Clifton Boulevard, Nottingham, NG7 2UH, U.K.

† For solvent keys, see Experimental.

Table 1. Properties of the esters isolated from gherkin hypocotyls

Ester	Colour in UV on paper* $-NH_3$	Colour in UV on paper* $+NH_3$	R_f values on 2D papers × 100 BAW	R_f values on 2D papers × 100 15% HOAc	Hydrolysis product
1	C	B	60	85	*p*-Coumaric acid
2	B	Gn	55	80	Ferulic acid
3	B	Gn	52	77	Caffeic acid

* B = blue, C = colourless, Gn = green.

No free phenolic acids were detected in dark-grown tissue but plants exposed to blue light for 4 hr contained free *p*-coumaric acid. Following 13 hr blue light, hypocotyls still contained some free *p*-coumaric acid, whilst no free phenolic acids were now detected in cotyledons.

In view of the difficulty in obtaining quantitative estimates using TLC and PC, and of the relatively low sensitivity of these techniques, hypocotyl phenolic acids and esters were analysed using HPLC, as detailed below. Since the contribution of *p*-coumaric acid to both the free phenolic acid and phenolic ester pools was large, relative changes in the magnitude of these pools were also obtained by monitoring the absorbance of extracts at 310 nm, the absorption maximum of *p*-coumaric acid in acid conditions. In acid conditions λ_{max}^{EtOAc} nm values in the range 270–350 nm are as follows: cinnamic acid, 280; *p*-coumaric acid 310; caffeic acid, shoulder 295, peak 325; ferulic acid, shoulder 295, peak 324. It therefore follows that measurement at 310 nm most severely underestimates cinnamic acid itself.

Changes in the concentrations of free cinnamic acids and cinnamic acid esters in hypocotyls treated with blue light

Free acids and esters were separated using DEAE-cellulose ion exchange chromatography [6]. The free acids were measured directly, while the esters were hydrolysed and the free acids formed extracted into EtOAc. Following exposure to blue light, the total concentration of 310 nm-absorbing material in hypocotyls in the combined free acid and ester fractions increased reaching a peak, at about 6 hr, of three times that in the dark control (Table 3), and then declining. The ratio of the ester pool to the free-acid pool was generally greater in the blue light-treated tissue, i.e. the free-acid pool contributed proportionately less to the total phenolic acid pool (Table 3).

HPLC analysis showed that both the free phenolic acid and the esterified phenolic acid pools contain cinnamic acid in addition to *p*-coumaric acid (Table 4). Great care was taken not to lose cinnamic acid since it was found that it had a tendency to come out of solution, having a very low solubility in acidic aqueous solutions, and to stick to glass containers. Extracts were never allowed to evaporate to dryness and the ion exchange cellulose was contained in a polycarbonate column. The presence of esterified caffeic acid in hypocotyls was confirmed (Table 4) and esterified ferulic acid was often, but not always, detected. The free-acid pool sometimes contained caffeic acid, in addition to *p*-coumaric acid and cinnamic acids, but free ferulic acid was rarely found.

Following exposure to blue light, the size of the free phenolic acid pool increased two-fold between 0 hr and 3.5 hr, reaching a plateau, but decreased between 6 hr and 13 hr to the dark level (Table 4). In a separate experiment it was found that no increase in free acids had occurred following 2 hr blue light. The esterified phenolic acid pool similarly increased following a 3.5 hr exposure to blue light, but continued to increase between 3.5 hr and 6 hr, reaching a peak at 6 hr (Table 4) which was *ca* 5- to 6-fold that of the dark level, between 6 hr and 13 hr. The ester pool decreased (Table 4) to a level still significantly higher

Table 2. Properties of the marker compounds

Marker phenolic acid	Colour in UV on paper* $-NH_3$	Colour in UV on paper* $+NH_3$	Colour in shortwave UV on fluorescent plate‡	R_f values × 100 BAW	CAW	15% HOAc	BzAW plate
Caffeic	PB	LB	D	78	48	46 + 60 + 76	06
Ferulic	B	B	D	82	80	52 + 76	31
p-Coumaric	C	P	D	88	73	60 + 80	28
Cinnamic	†D	†D	D	95	88	74 + 86	46

* Observed under 360 nm light.
† Observed under 254 nm light only.
‡ Observed under 254 nm light.
B = blue, C = colourless, D = dark brown/black, L = light, P = purple.

Table 3. Total A_{310nm} in hypocotyl tissue exposed to blue light

Treatment	Time (hr)	Free	Ester	Total	Ester/free
Dark	0	0.57	2.23	2.80	3.9
Blue	1.5	0.52	2.10	2.60	4.1
Blue	3.5	0.77	3.90	4.70	5.1
Blue	6.0	0.76	6.40	7.10	8.4
Dark	6.0	0.50	1.58	2.08	3.1
Blue	13.0	0.47	4.85	5.30	7.6
Dark	13.0	0.38	1.73	2.11	4.5

EtOAc extracts measured in 1 cm pathlength cells and units expressed per g fr. wt of tissue. Ester fraction hydrolysed with NaOH prior to extraction with EtOAc.

than that of the dark control. In dark-grown hypocotyls the free pool contained *p*-coumaric and cinnamic acids in a ratio of approximately 2:1; blue light-treated tissue contained a smaller proportion of cinnamic acid relative to *p*-coumaric acid, particularly following 3.5 hr blue light (Table 4). The contribution of cinnamic acid to the ester fraction was more variable, but was generally greater in dark than in the blue light-treated hypocotyls. The free phenolic acid pool generally contributed less to the total acid pool in the light than in the dark, confirming the A_{310} results.

Identification of the sugar component of the p-*coumaryl ester*

The *p*-coumaryl ester extracted from hypocotyls was isolated by means of preparative PC (see Experimental), acid-hydrolysed, extracted with EtOAc and the remaining aqueous layer used for the determination of the sugar component by PC. The sample exhibited the same properties as the glucose marker in three solvents; the ester is thus confirmed as *p*-coumaroylglucose. The R_f data of the other two acid esters (Table 1) are consistent with them also being the respective glucose esters.

DISCUSSION

The present work represents one of the first reports of the existence of measurable amounts of free cinnamic acid in healthy plant tissue and it highlights the problems and variations encountered when dealing with this acid. The successful detection of cinnamic acid was due to the use of HPLC, since it is not easily detected by PC and TLC.

In addition to cinnamic acid, both the free acid and esterified acid pools contain *p*-coumaric acid; in blue light, caffeic acid is sometimes also present and, less frequently, ferulic acid. In previous work on the phenolic acids of gherkin seedlings [5], when the extracts were alkali-hydrolysed prior to analysis, *p*-coumaric acid was the major phenolic acid detected; ferulic acid was also detected, but not caffeic and cinnamic acids. The failure to detect cinnamic acid is not surprising and it is possible that caffeic acid was identified as ferulic acid since these acids have very similar properties on the 1D PC systems employed in this earlier work.

The present work was concerned with changes in phenolic acid composition in hypocotyls following treatment with blue light, though similar results were obtained with cotyledon tissue. Blue light doubles the total concentration of cinnamic acid and *p*-coumaric acid in the free-acid pool during a 3.5 hr period and causes a slightly larger increase in the ester pools, which are presumably derived from the free-acid pool. The largest increase in both pools is in *p*-coumaric acid. Both this and cinnamic acid are known to inhibit the activity of PAL in gherkin tissues following excision [2, 7] and, in the case of cinnamic acid, at least, this is partly an effect on PAL synthesis [7]. Since the increase in PAL activity in response to blue light occurs during a period of 3.5 hr in which there is an increase in both acids, it seems unlikely that the increase is a consequence of feedback control by the phenolic acids and must be caused in some other way. Alternatively, it is possible that blue light causes a conversion of *trans*-(hydroxy)-cinnamic acids into the

Table 4. HPLC analysis of phenolic acids in the free acid- and esterified acid pools of hypocotyl tissue exposed to blue light

Treatment	Time (hr)	Free (μg/g fr. wt)					Esters (μg/g fr. wt)					Overall total
		p-Coum.	Cinn.	Caff	Fer.	Total	*p*-Coum.	Cinn.	Caff.	Fer.	Total	
Dark	0	24.0	12.0	0	0	36.0	30.4	0	1.2	0	31.6	67.6
Blue	3.5	61.5	5.2	2.8	0	69.5	46.4	0	2.6	0	49.0	118.5
Blue	6.0	53.5	10.9	0	0	64.4	78.6	35.0	33.0	0	146.6	211.0
Dark	6.0	22.9	13.6	0	0	36.5	6.9	20.9	0	0	27.8	84.3
Blue	13.0	22.0	5.0	0	0	27.0	58.0	0	12.7	0	68.7	95.7
Dark	13.0	25.7	11.0	0	0	36.7	7.0	15.0	0	3	25.0	61.7

much less inhibitory *cis* isomers, as suggested by Engelsma [8], which might be expected to lead to an increase in PAL synthesis as well as enhanced catalytic activity. However, the absence of any incorporation of density-label into the enzyme during a 4 hr induction period [9] suggests that a stimulation of PAL synthesis is not a major contributor to the increase. The increase in both free-acid and ester pools of phenolic acids is consistent, however, with the hypothesis [7] that these compounds could be the cause of the decline in PAL activity between 3.5 hr and 6 hr, both by feedback repression of PAL synthesis [7] and possibly by inducing the formation of a PAL–inhibitor complex [10].

In addition to having a repressive effect on the induction/stimulation of PAL activity, cinnamic acid is a competitive inhibitor of the catalytic activity of PAL in gherkins [11]. Changes in free cinnamic acid concentration could therefore directly modulate the rate of the PAL reaction *in vivo*. Although the concentration of phenylalanine in gherkin tissue was not measured, free unbound phenylalanine is generally as low as 0.1–0.2 μmol/g fr. wt in plant tissues [12]. This compares with cinnamic acid at a concentration of *ca* 0.08 μmol/g fr. wt in dark gherkin tissues, and 0.03 μmol/g fr. wt following 3.5 hr blue light. Thus, if the phenylalanine concentration in gherkins is as low as 0.1 μmol/g fr. wt, then cinnamic acid, at least in the dark, would have some inhibitory effect on PAL activity *in situ*. It is worth noting that the other acids detected, *p*-coumaric, ferulic and caffeic acids, are not inhibitors of PAL activity [11] and, in addition, *p*-coumaric acid is not an inhibitor of CA4Hase activity [13]. On the other hand, *p*-coumaric acid does prevent a stimulation in extractable CA4Hase activity under certain conditions [3, 4].

EXPERIMENTAL

Plant material. Gherkin seedlings (*Cucumis sativus* cv Venlo Pickling, Suttons, U.K.) were grown in darkness at 25° ± 1° for 9 days in sealed plastic (22 × 11 cm) boxes on tissue paper. They were pretreated with blue light (40 W blue fluorescent tubes (Thorn Ltd) filtered through 1 layer of No. 3-Cinemoid (Rank-Strand Electric Ltd.); 3 W/m²; 15 μmol/m²) before extraction [8]. Cotyledons or the upper 1.5 cm of hypocotyls including plumular hook were used.

Isolation procedures for HPLC analysis. Tissue (*ca* 5 g) was frozen in liquid N_2, powdered using a pestle and mortar and extracted with boiling 80% EtOH (100 ml) for a total of 20 min. After cooling to room temp. the extract was centrifuged at 30 000 *g* for 15 min. The supernatant was concd *in vacuo* to *ca* 10 ml and centrifuged again at 30 000 *g* for 15 min before loading on to DEAE-cellulose (Whatman) in a polycarbonate column (4.5 cm long; 1.5 cm i.d.) pre-equilibrated with 0.01 M acetate buffer (pH 6.0) [6]. The column was washed with this buffer (*ca* 150 ml) at a flow of 1.5 ml/min. The eluted fraction contained the sugar esters. The phenolic acids were diluted with 50 ml 0.1 M H_2SO_4 (15 ml/min). Using this method the recovery of phenolic acid standards from the column was >90%. Both ester and free acid fractions were concd *in vacuo* (esters to *ca* 6 ml, acids to *ca* 3 ml) but were *never* allowed to dry completely. Esters were then hydrolysed in 2 M NaOH for 2 hr at room temp. and acidified with conc. HCl. Acids from both the hydrolysed ester and free acid fractions were extracted into distilled EtOAc (3 × 3 ml); all extracts were scanned on an SP 1800 spectrophotometer between 220 nm and 370 nm and recovery in the first EtOAc extract calculated using A_{310nm}. The first EtOAc extract was analysed by HPLC and TLC. No esters were detected in the free acid fraction.

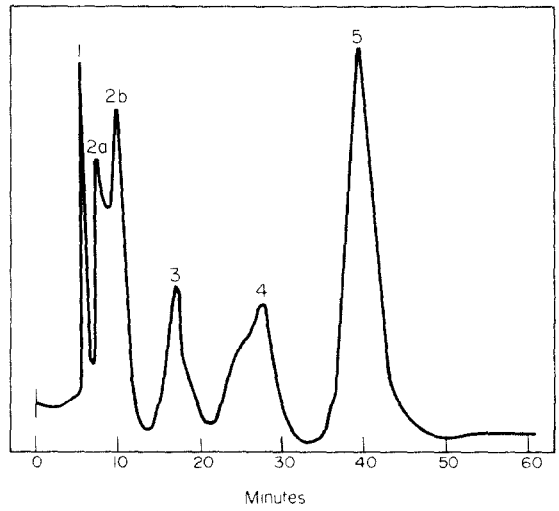

Fig. 1. Separation of phenolic acids on Partisil 10 ODS-2 with HOAc–H_2O (1:9). 1 = solvent front; 2a and 2b = *cis*- and *trans*-caffeic acid; 3 = *p*-coumaric acid; 4 = ferulic acid; 5 = cinnamic acid. Flow rate *ca* 1 cm³/min.

HPLC analysis. A 25 cm long column of i.d. 4.6 mm packed with Partisil 10 ODS-2 reversed-phase material (Whatman, 10 μm) was used in an Applied Research Labs apparatus (LC 750) equipped with a 254 nm UV detector and a Specac 30 100 Injection Valve (Analytical Accessories, Orpington, Kent). Fifty μl quantities of extracts and standards were injected and chromatographs at pressures of 800–1200 psi which gave a flow rate of 1 ml/min. The solvent was HOAc–H_2O (1:9). The column was calibrated frequently using 2 or 4 mg of re-crystallized samples of standard phenolic acids in EtOAc. The concn of each phenolic acid was proportional to the peak height, which of course was different for each acid since the detector was monitoring at 254 nm (see Fig. 1).

Isolation procedure for TLC and PC analysis. Whole tissue was boiled in 80% MeOH (2 g/20 ml) for 10 min and left at room temp. for 24 hr. The supernatant was evapd *in vacuo* and the residue dissolved in 0.2 ml 70% EtOH. 20 ml of this ethanolic extract was fractionated by 2D PC; the rest was evapd almost to dryness, dissolved in 0.5 ml H_2O, acidified with 2 N HCl and the free acids extracted immediately into EtOAc. The free acid extract was evapd in a cold air stream, dissolved in 0.1 ml 95% EtOH and 2 ml analysed by TLC. Esters in the acidified sample were hydrolysed by boiling for 30 min, extracted into EtOAc, evapd in a cold air stream, dissolved in 0.2 ml 95% ethanol and 20 ml aliquots analysed by PC and TLC.

TLC and PC analysis. For 2D PC, samples were spotted on Whatman No. 1 paper (23 × 28 cm) and developed using *n*-BuOH–HOAc–H_2O (4:1:5, upper phase, BAW) in the first direction, and 15% HOAc in the second direction. PC was also performed in one dimension using BAW, $CHCl_3$–HOAc–H_2O (30:15:2, CAW) or 15% HOAc as solvent. To facilitate detection of cinnamic acid, extracts were also chromatographed on plastic fluorescent 0.25 mm Si gel plates (Camlab, Cambridge) using C_6H_6–HOAc–H_2O (2:2:1, upper phase, BzAW). Sugars were analysed by PC using 57 cm long paper and running by descent in BAW, *n*-BuOH–C_6H_6–C_5H_5N–H_2O (5:1:3:3, BBPW) or PhOH–H_2O (4:1). The standards used included glucose, galactose, arabinose, xylose, rhamnose and glucuronic acid; detection was by means of aniline hydrogen phthalate [14].

Preparative PC. To isolate the *p*-coumaryl ester, concentrated ethanolic extracts were chromatographed as streaks on sheets of

Whatman No. 3 mm (46 × 57 cm) paper, run by descent in BAW. A band which became purple on fuming with NH_3 (R_f 0.65) was diluted with 80% MeOH for 24 hr, evapd to dryness, dissolved in 70% EtOH and chromatographed as a streak in 15% HOAc. The band which became purple on fuming with NH_3 was diluted into 80% MeOH as before, dried, hydrolysed in 1.5 N HCl, and extracted into EtOAc. The acid component (EtOAc fraction) was analysed by 1 D PC and TLC whilst the sugar component (aq. fraction) was analysed by PC (see above), in both cases after further concentration, and identified as glucose.

Acknowledgement—The support of the SRC in providing a Research Grant to C. B. Johnson is gratefully acknowledged.

REFERENCES

1. Hanson, K. R. and Havir, E. A. (1979) in *Recent Advances in Phytochemistry. Biochemistry of Plant Phenolics* (Swain,, T., Harborne, J. B. and Van Sumere, C. F., eds.) pp. 91–137. Plenum Press, New York.
2. Engelsma, G. (1968) *Planta (Berlin)* **82,** 355.
3. Durst, F. (1976) *Planta (Berlin)* **132,** 221.
4. Billett, E. E. and Smith, H. (1981) *Phytochemistry* **20,** (in press).
5. Engelsma, G. and Meijer, G. (1965) *Acta. Bot. Neerl.* **14,** 54.
6. Molderez, M., Nagels, L. and Parmentier, F. (1978) *Phytochemistry* **17,** 1747.
7. Johnson, C. B., Attridge, T. H. and Smith, H. (1975) *Biochim. Biophys. Acta* **385,** 11.
8. Engelsma, G. (1974) *Plant Physiol.* **54,** 702.
9. Attridge, T. H. and Smith, H. (1974) *Biochim. Biophys. Acta* **343,** 452.
10. Billett, E. E., Wallace, W. and Smith, H. (1978) *Biochim. Biophys. Acta* **524,** 219.
11. Iredale, S. E. and Smith, H. (1974) *Phytochemistry* **13,** 575.
12. Margna, U. (1977) *Phytochemistry* **16,** 419.
13. Billett, E. E. and Smith, H. (1978) *Phytochemistry* **17,** 1511.
14. Ribereau-Gayon, P. (1972) *Plant Phenolics.* Oliver & Boyd, Edinburgh.