HYDROXYLATION OF *p*-COUMARIC ACID BY ILLUMINATED CHLOROPLASTS FROM SPINACH BEET LEAVES

D.J. BARTLETT, J.E. POULTON and V.S. BUTT*
Botany School, Oxford University, South Parks Road, Oxford OX1 3RA, England

Received 27 March 1972

1. Introduction

The hydroxylation of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid (3,4-dihydroxycinnamic acid) is a major reaction in the conversion of L-phenylalanine to lignin and many flavonoids in plants. The reaction has been studied in some detail using a purified phenolase from leaves of spinach beet [1,2]; it required both molecular oxygen and reducing agent. Ascorbate, NADH, NADPH, tetrahydrofolate and 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (dimethyltetrahydropteridine) were all effective as reducing agents.

Losses during purification of the crude enzyme suggested that the enzyme was firmly bound to particles, from which it could be released only by prolonged exposure to $(NH_4)_2SO_4$. Kenten [3,4] showed that the phenolase of broad bean leaves was brought into solution only by treatment with acid or alkali or by anionic detergents, and the phenolase of sugar beet leaves was released from chloroplast preparations only with acetone or deoxycholate [5]. Distribution studies of the phenolase of spinach beet leaves reported here have shown the enzyme to be associated mainly with chloroplasts, firmly bound to the lamellae.

Sato [6] demonstrated the hydroxylation with chloroplasts from leaves of a number of plants, without any added reducing agent. These observations have been examined in more detail using preparations of intact chloroplasts [7] to show that the reaction is promoted by illumination with the same essential requirements under these conditions as with the extracted enzyme.

* To whom to address all correspondence.

2. Materials and methods

2.1. *Distribution of enzyme activity*

Homogenates of freshly-harvested leaves from plants of spinach beet (*Beta vulgaris* L. ssp. vulgaris), grown in the greenhouse under artificial illumination or in the garden, were fractionated by the procedure of Tolbert et al. [8]. 100 g of washed leaves, from which the larger veins and midribs had been removed, were homogenised with 200 ml of 0.5 M sucrose containing 20 mM glycylglycine buffer pH 7.5 in a Waring Blendor for about 1 min. The macerate was squeezed through muslin and centrifuged at 0–2° successively at 500 *g* for 25 min, 6,000 *g* for 20 min and finally 30,000 *g* for 20 min. Each precipitate was resuspended in 10–15 ml of the homogenising medium and, with the final supernatant, assayed for hydroxylase and catechol oxidase activities [1].

2.2. *Experiments with illuminated chloroplasts*

100 g of the washed laminae of leaves were macerated in a Waring blendor with 200 ml of ice-cold 0.33 M sorbitol, containing 0.01 M sodium pyrophosphate buffer pH 6.5. The macerate was squeezed through muslin and the liquid centrifuged at 500 *g* for 25 min, and then at 6,000 *g* for 20 min to sediment whole chloroplasts. The precipitate was resuspended in the extraction medium, and again sedimented at 6,000 *g* for 20 min. This fraction was finally suspended in 10–15 ml of homogenising medium.

Aliquots of the preparation, containing 0.25–1.5 mg of chlorophyll, were incubated with *p*-coumaric acid (10 μmoles) and 1.0 ml of HEPES buffer pH 7.6 at 12–15° in a total vol of 3 ml, with shaking, under an illumination of 1,200 ft-candles. The reaction was

stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid, and caffeic acid determined [1].

2.3. *Chlorophyll estimation*

The chlorophyll content of fractions was estimated spectrophotometrically [9].

3. Results

3.1. *Distribution of phenolase activity*

The hydroxylation activities sedimented from the homogenate were not concentrated in any single fraction (table 1). The fractions precipitated at 500 g and 6,000 g and likely to be richest in intact chloroplasts, contained less total activity than either the fraction sedimented at 30,000 g or the final supernatant. No relation between the fractions could be seen when these results were expressed on a protein basis, but the specific activities, determined in relation to their chlorophyll content, were all very similar. It is concluded that the enzyme was firmly bound with the chlorophyll to the chloroplast lamellae, so that the enzyme was distributed with chloroplast fragments in the other fractions. A significant amount of unattached enzyme appears to have been present in the supernatant fraction, perhaps released during the homogenisation and fractionation in glycylglycine buffer [10, 11]. A peroxisomal preparation, separated from the chloroplast fraction (6,000 *g* precipitate) by sucrose-density centrifugation [8] showed no hydroxylase activity.

A similar distribution pattern was observed for catechol oxidase activity. The differences between the ratios of hydroxylase to catechol oxidase activities in the four fractions (0.19, 0.20, 0.25 and 0.19, respectively) and the homogenate (0.17) are not considered to be significant so that no other phenolase activities were present in any of the fractions in appreciable quantity.

3.2. *Hydroxylation of p-coumaric acid by illuminated chloroplasts*

Illuminated chloroplast suspensions hydroxylated *p*-coumaric acid to caffeic acid without added reductant at a rate similar to that observed in darkness when ascorbate was provided (fig. 1). However, whereas the rate of the reaction in darkness began to decline after 30 min, perhaps as the ascorbate became exhausted [1], it remained linear over 60 min when the chloroplasts were illuminated.

The amount of caffeic acid produced by illuminated chloroplasts was over three times as much as in darkness, when no reductant was added (table 2). Some endogenous reductant may have been responsible for the production of caffeic acid in darkness; this was apparently regenerated in light by reactions sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The importance of oxygen in the reaction was shown by its inhibition in an anaerobic atmosphere in light so that even less caffeic acid was produced than in darkness.

Table 1

Distribution of *p*-coumaric hydroxylase and catechol oxidase activities in fractions from spinach beet leaf homogenate.

Fraction	Hydroxylase		Catechol Oxidase	
	Specific activity (m-units/ mg chlorophyll)	Total activity (m-units)	Specific activity (m-units/ mg chlorophyll)	Total activity (m-units)
Crude homogenate	210	7182 (100%)	1233	42180 (100%)
500 *g* precipitate	205	1577 (22.0%)	1081	8338 (19.8%)
6,000 *g* precipitate	170	998 (13.9%)	867	5085 (12.6%)
30,000 *g* precipitate	180	2704 (37.6%)	715	10733 (25.4%)
30,000 *g* supernatant	618	2647 (37.0%)	3197	13699 (32.5%)

The figures in parentheses indicate the activity of each fraction as a percentage of crude homogenate.

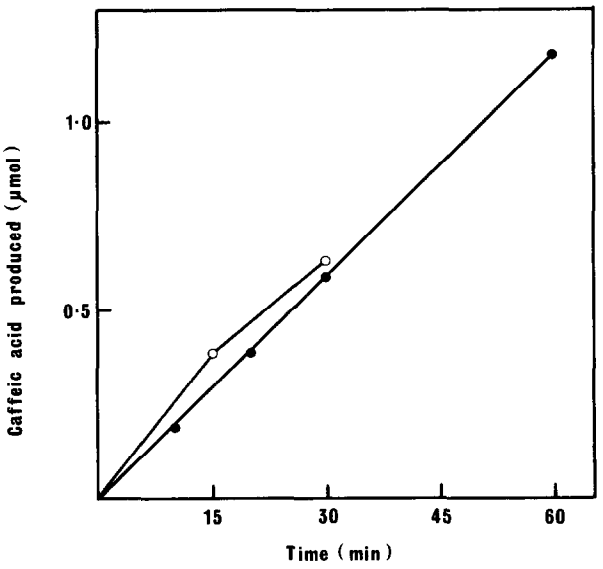

Fig. 1. Time course of caffeic acid production by chloroplast preparations in light (●–●–●), and in darkness with addition of 10 μmole ascorbate (○–○–○). The conditions were those described for table 2, with samples taken after the periods indicated.

4. Discussion

The results suggest that the enzyme is bound to chloroplast lamellae and, in the catalysis of *p*-coumaric acid hydroxylation, requires oxygen and a reductant, which appears to be regenerated in light, Attention has been drawn to the special role of dimethyltetrahydropteridine in suppressing additional catechol oxidase activity during hydroxylation [12], and its likely participation in chloroplast oxidoreductions [13] suggests that it may act here as the reductant. The possible participation of ferredoxin or reduced plastoquinone as primary reductants produced by the two photosystems in chloroplasts needs investigation.

Table 2
Hydroxylation of *p*-coumaric acid in chloroplast suspensions.

Conditions	Caffeic acid produced (μmole)
Light, air	0.45
Dark, air	0.12
Dark + ascorbate (10 μmoles), air	0.42
Light + DCMU (1 μM), air	0.13
Light, anaerobic	0.07

0.5 ml of chloroplast suspension (containing 0.63 mg of chlorophyll) was incubated with 1.0 ml of 0.05 M HEPES buffer, pH 7.6, together with additions where shown, in a total vol of 3 ml at 12° with shaking. *p*-Coumaric acid (10 μmoles) was added after 30 min illumination, where indicated, and incubation continued for a further 30 min, when caffeic acid was determined.

References

[1] P.F.T. Vaughan and V.S. Butt, Biochem. J. 113 (1969) 109.
[2] P.F.T. Vaughan and V.S. Butt, Biochem. J. 119 (1970) 89.
[3] R.H. Kenten, Biochem, J. 67 (1957) 300.
[4] R.H. Kenten, Biochem. J. 68 (1958) 244.
[5] A.M. Mayer and J. Friend, Nature 135 (1960) 464.
[6] M. Sato, Phytochemistry 5 (1966) 385.
[7] W. Cockburn, D.A. Walker and C.W. Baldry, Biochem. J. 107 (1968) 89.
[8] N.E. Tolbert, A. Oeser, T. Kisaki, R.H. Hageman and R.K. Yamazaki, J. Biol. Chem. 243 (1968) 5179.
[9] D.I. Arnon, Plant Physiol. 24 (1949) 1.
[10] N.E. Tolbert, A. Oeser, R.K. Yamazaki, R.H. Hageman and T. Kisaki, Plant Physiol. 44 (1969) 135.
[11] R. Parish, European J. Biochem. 22 (1971) 423.
[12] P.F.T. Vaughan and V.S. Butt, Biochem. J. 127 (1972) 641.
[13] R.C. Fuller and N.A. Nugent, Proc. Natl. Acad. Sci. U.S. 63 (1969) 1311.