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BETACYANIN DECOLOURIZING ENZYME IN *AMARANTHUS TRICOLOR* SEEDLINGS

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Key Word Index—*Amaranthus tricolor*; Amaranthaceae; decolourizing enzyme; cytokinins; betacyanins.

Abstract—The characterization and partial purification of an enzyme from *Amaranthus tricolor* which decolourizes betacyanin are described. The enzyme occurs in greatest amounts in the roots and in 3.5–4-day-old seedings. Preparation from an acetone powder of roots results in a more active and more stable enzyme than that obtained from crude buffer extraction. The activity is in the 130 000 *g* supernatant from sucrose–buffer extraction. It has a pH optimum of 3.4, K_m towards amaranthin of 3.1×10^{-6} M and towards betanin of 3.5×10^{-6} M, and is inhibited by lack of oxygen, and by azide, diethyldithiocarbamate, thiourea, dithiothreitol and cysteine. The product of the reaction has the spectral and electrophoretic properties of betalamic acid. The possibility of enzymic decolourization of betacyanin during acetic acid extraction used for assay of the pigment in the *Amaranthus* bioassay for cytokinins needs to be recognized.

INTRODUCTION

Betacyanins are red-violet pigments characteristic of most members of the order Centrospermae [1, 2]. The accumulation of the betacyanin, amaranthin, in *Amaranthus tricolor*, has been used as the basis for a bioassay for cytokinins [3]. In this bioassay it has been observed that the colour of 3 mM acetic acid extracts fades with time [Elliott, D. C., unpublished observations]. The possibility that this fading was due to an enzymic reaction has now been investigated, and comparisons made between the enzyme found and a betacyanin decolourizing enzyme from red beet tissue [4, 5].

RESULTS

In most experiments the betacyanin from betroot, betanin, was used as substrate, with some key results being repeated with amaranthin isolated from cytokinin-induced *A. tricolor* seedlings.

A survey of seedlings of different ages is shown in Table 1. It is clear that 3.5–4-day-old seedlings contain the highest decolourizing specific activity and the greatest amount per seedling. Seedlings were, therefore, used after 88 hr germination at 25° for all further experiments reported in this paper. The root was the richest source of activity. Comparison of mixing experiments, with root and shoot extracts, indicated that the low activity in the shoot was probably due to the presence of an inhibitor (Table 2). Acetone powder extracts did not contain this inhibitor.

The pH optimum of the enzyme was 3.4 as found by Soboleva *et al.* [4], the shape of the pH curve also being very similar (results not shown). However, a major difference from the other workers [4, 5] was that the enzyme isolated from *Amaranthus* seedlings was soluble (Table 3).

Partial purification of the enzyme from the 48 000 *g* supernatant could be achieved by ammonium sulphate fractionation (50–100% saturation). However preparation of a buffer extract from an acetone powder resulted in a more active and more stable enzyme (Table 4).

The enzyme activity exhibited a Q_{10} of 2.0 from 25° up to 37°. An incubation temperature of 25° was chosen

Table 1. Effect of seedling age on decolourizing enzyme activity

Age of seedlings (days)	Enzyme activity		
	pkat/seedling	pkat/g fr.wt	pkat/mg protein
2.5	0.57	158.7	49.59
3.0	0.77	180.7	58.63
3.5	0.81	167.5	92.57
4.0	0.76	145.5	86.62
5.0	0.67	98.8	61.0

Whole seedlings were extracted and the 48 000 *g* supernatant assayed. Data is for assays performed with 0.1 ml extract, equivalent to 0.025 g fr. wt and with protein content of 80–40 μg, depending on seedling age.

Table 2. Distribution of decolourizing enzyme in 3.5-day-old seedlings

Source of enzyme	Vol. (μl)	Protein (μg)	Enzyme activity (ΔA_{530}/5 min)
Buffer extract of fresh material			
Root	100	25	0.032
	250	63	0.045
Shoot	100	100	0.031
	250	250	0.029
Root + shoot (100 μl each)	200	125	0.047
Acetone powder extracted in buffer*			
Root	100	11	0.021
	250	28	0.055
Shoot	100	38	0.036
	250	95	0.060
Root + shoot (100 μl each)	200	49	0.063

*Acetone powders were prepared by grinding frozen material to a powder and extracting with four vols. acetone (−20°) in a mortar and pestle. After filtering and washing with acetone (1 vol.) the powder was dried under vacuum for 30 min, then extracted with 0.1 M citrate buffer, pH 3.4 (0.3 g original fr. wt/ml) using a glass–Teflon homogenizer at 4°, and centrifuged at 48 000 *g* for 60 min to remove debris and insoluble material.

Table 3. Sub-cellular fractionation of decolourizing enzyme

Fraction*	Specific activity (pkat/mg protein)	Total activity (pkat)
1000 *g* pellet	0	0
12 000 *g* pellet	1.4	3.7
130 000 *g* pellet	0.7	3.7
130 000 *g* supernatant	50.8	200.9

*Roots from 3-day-old seedlings (harvest from 1 g dry wt seeds) were homogenized in 0.5 M sucrose in 0.5 M Tris–HCl buffer, pH 7.0 (0.3 g fr. wt/ml). The pellet from 100 *g* (10 min) was discarded. Pellets from 1000 *g* (10 min), 12 000 *g* (30 min) and 130 000 *g* (60 min) centrifugations were resuspended in 0.1 M citrate buffer, pH 3.4 (vol. ≡ 0.5 × original fr. wt). The 130 000 *g* supernatant was freed of small MW components by gel filtration through Sephadex G-25, 0.1 M citrate buffer, pH 3.4, being used for equilibration and elution.

because this was the germination temperature. Pre-incubation at 50° for 5 min destroyed most of the activity (Table 5). The K_m for amaranthin was 3.1×10^{-6} M and for betanin 3.5×10^{-6} M. These values were obtained using a 48 000 *g* supernatant. The enzyme response curve for a buffer extracted acetone powder showed a linear relationship up to 50 μg protein in the standard assay. In a 48 000 *g* supernatant from a crude buffer extract of fresh roots a lag phase of 1–2 min was usually observed with amounts of enzyme < 350 μg. A plot of the linear rates measured after this time against enzyme concentration showed a linear relationship only up to 125 μg protein per assay. The lag phase appeared to be a property of the substrate–enzyme ratio since it was proportional to the substrate concentration if the enzyme concentration was kept constant. A preparation of β-glucosidase from almonds (Sigma) had no effect on the lag phase or the rate of reaction, indicating that deglycosidation is not a pre-requisite for decolourization.

Table 4. Partial purification of decolourizing enzyme by ammonium sulphate fractionation and by acetone precipitation, and stability of preparations

Fraction	Protein per assay (μg)	Specific activity (pkat/mg protein)	Enzyme activity (as % of original activity) after 4 days at 2°	−15	−85
Method 1*					
48 000 *g* supernatant	370	46.1	132	—	—
0–50% $(NH_4)_2SO_4$ pellet	18	11.0	—	—	—
50–100% $(NH_4)_2SO_4$ pellet	56	74.4	0	1.5	46
50–100% $(NH_4)_2SO_4$ supernatant	12	15.0	—	—	—
Method 2†					
Acetone powder	135	100.2	30	46	68

*Roots from 3.5-day-old seedlings were extracted as described in the Experimental to give a 48 000 *g* supernatant which was fractionated with ammonium sulphate at 4°, the precipitates taken up in 0.1 M citrate buffer, pH 3.4, and dialysed against the same buffer overnight.

†Acetone powder of roots from 3.5-day-old seedlings was prepared and treated as in Table 2.

Table 5. Effect of pre-treating decolourizing enzyme at different temperatures

Conditions	Enzyme activity (pkat/mg protein)
Control	45.5
Boiled enzyme	0.7
Enzyme pre-treated at 35° for 5 min	43.4
Enzyme pre-treated at 50° for 5 min	4.8

The 48 000 *g* supernatant of roots from 3.5-day-old seedlings was prepared as described in the Experimental. Assays were performed at 25°.

The effect of anaerobiosis on the rate of reaction is shown in Table 6. As found by Soboleva *et al.* [4], although not to the same extent, anaerobic conditions inhibit. The effects of various inhibitors of metal-containing enzymes and sulphydryl reagents are shown in Table 7. As seen for the beet enzyme [4], inhibitors of metal-containing enzymes were markedly effective.

Fig. 1 shows a time–wavelength scan of decolourization of betanin. The reaction product gave a λ_{max} *ca* 434 nm which indicated betalamic acid [7], while calculation of the stoichiometry using ε_{537} for betanin of 6.05×10^4 [8] and ε_{434} for betalamic acid of 2.3×10^4 [7] gives 5.77 nmol of betalamic acid appearing for the loss of 6.92 nmol betanin. Other tests for betalamic acid were also positive, e.g. increased A on mixing with aniline (λ_{max} 420 nm). After freeze-drying, the reaction mixture from a standard assay was electrophoresed in pyridine–acetic acid buffer, pH 6.5, and a yellow spot had a mobility relative to betanin of 0.19 as reported for betalamic acid by Kimler *et al.* [9].

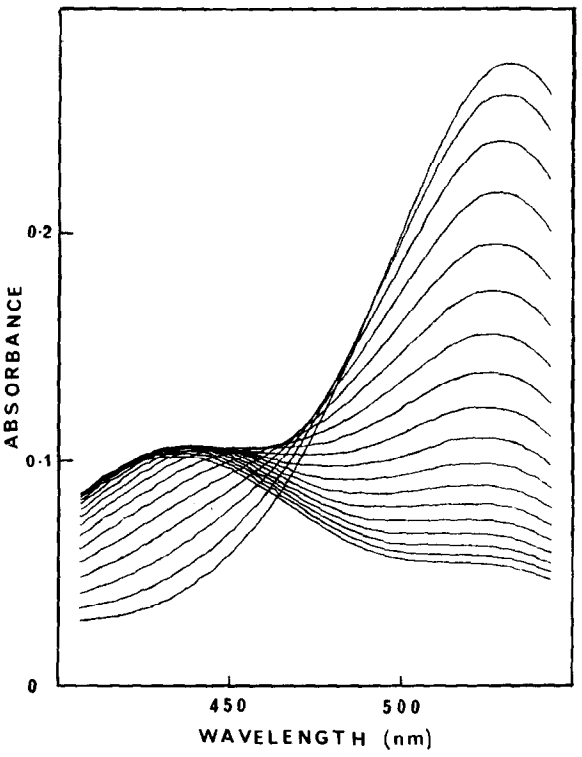

Fig. 1. Time–wavelength scan of decolourization of betanin. The cycle delay time was 0 and the scan speed 120 nm/min, therefore each successive scan was separated by 1.25 min.

Table 6. Effect of anaerobiosis on decolourizing enzyme activity

Conditions	ΔA_{537} in 30 min
Air	0.171
Vacuum	0.089

Assay contents were identical to those used in the rate assay method. Reaction mixtures were incubated either using aerated buffer in test tubes (air) or in Thumberg tubes after evacuation and gassing with nitrogen twice, ending with evacuation (vacuum).

Since control of the accumulation of betacyanin is shown by cytokinins, the effect of this hormone on decolourizing enzyme activity *in vitro* and on the level of enzyme extracted from seedlings pre-treated with hormone were investigated. However, no significant effects were observed.

Table 7. Effect of inhibitors on decolourizing enzyme activity

Inhibitor	Concentration for half-maximal activity (mM)	Action [6]
Diethyldithiocarbamate	0.1	Irreversible inhibition of copper enzymes at 10^{-3} M. May also react with Zn^{2+}, Fe^{2+} and other metalloenzymes. Unstable in acid conditions.
Sodium azide	0.25	Inhibits iron enzymes at 10^{-3} M; uncouples phosphorylation at 10^{-4} M. Active form un-dissociated, pK_a, 4.72.
Cysteine	0.5	Reducing agent
Dithiothreitol	0.5	Reducing agent
Thiourea	2.0	Forms soluble complexes with heavy metals especially Cu^{2+}. Fairly strong reducing agent
α, α′-Dipyridyl	Activates at 0.5	Fe^{2+} chelator; may inhibit Fe^{2+} enzymes at 10^{-8} M.

DISCUSSION

The decolourizing enzyme present in *A. tricolor* seedlings is clearly a factor to be reckoned with in assaying betacyanin accumulated in the bioassay for cytokinins [3]. Since extraction with 3.33 mM acetic acid results in an extract of pH *ca* 5, where the decolourizing enzyme is still active, care must be taken to keep the extract cold and to read the colour as soon as possible.

It may be significant that the highest concentration of the decolourizing enzyme is found in the roots. The work of Kojima *et al.* [10] shows that the cyanogenic glucoside, dhurrin, is located entirely in the epidermal layers of the leaf blade of *Sorghum bicolor*, whereas the enzymes responsible for its catabolism, a β-glucosidase and hydroxynitrile lyase, reside almost exclusively in the mesophyll tissue. In *Amaranthus tricolor*, where betacyanin accumulation in 3.5-day-old seedlings, in response to cytokinin or phytochrome, is confined to the lower epidermis of the cotyledon or the endodermis of the hypocotyl [11], it has not yet been possible to separate protoplasts of mesophyll and epidermis to investigate the distribution of decolourizing enzyme in cotyledons. A protective mechanism suggested by Kojima *et al.* [10], whereby the degradative activity was controlled by endogenous inhibitors (in the cotyledons), may be the case since there was evidence of this in mixing experiments.

The possibility that cytokinin stimulation of betacyanin accumulation was via a repression of decolourizing enzyme rather than increase in the synthetic pathway [11] does not appear to be likely since no effect of benzyladenine on the decolourizing enzyme either *in vitro* or *in vivo* was observed.

The conclusion of Soboleva *et al.* [4] that the enzyme was a polyphenoloxidase, given prior deglycosidation, was based mainly on inhibitor results. Our results are similar with the inhibitors of metal-containing enzymes although not as marked with anaerobiosis. It is not, however, necessary to involve oxidation at the 3,4-position of the cyclodopa ring, since hydrolysing the molecule between the cyclodopa and dihydropyridine rings as in Fig. 2 can explain the spectral changes (Fig. 1) seen during the enzyme reaction.

EXPERIMENTAL

Amaranthus tricolor seeds were germinated at 25° for 88 hr under standard conditions [3]. Enzyme extracts were prepared by homogenizing plant material in 0.1 M citrate buffer, pH 3.4 (0.3 g fr. wt/ml), using a mortar and pestle at 4°. After filtering through four layers of muslin the extract was centrifuged at 100 *g* for 5 min and the pellet discarded. Unless stated otherwise the 100 *g* supernatant was centrifuged at 48 000 *g* for 60 min and the clear supernatant used.

The decolourizing enzyme was assayed by following the initial rate of change of *A* at 537 nm at 25°. The reaction mixture (2 ml) contained enzyme (50–125 μg protein in 0.1 M citrate buffer, pH 3.4), 0.5 ml 0.1 M citrate buffer, pH 3.4, 1 ml substrate (10 nmol in the same buffer). Protein was estimated by the Lowry method [12], with BSA as standard.

Betanin was isolated from commercially grown *Beta vulgaris* with some modifications of published methods [13–15]. Beet juice was extracted (1 g clean diced root/2 ml H_2O) using a commercial food processor (Sanyo homogenizer on 'Puree' setting for 30 sec), the homogenate being filtered through three layers of muslin and the solid material then reprocessed and filtered. The combined juice fractions were lyophilized [13], solids resuspended in H_2O to give 100 mg/ml and then heated at 85° for 10 min [14]. After cooling the pH was adjusted to 2 with conc. HCl, centrifuged at 12 000 *g* for 30 min and the supernatant again lyophylized [15]. The resultant powder was resuspended in 0.1 M citrate buffer, pH 3.4, applied to a column of Sephadex G-25 [15] equilibrated with the same buffer and the pink fractions collected, combined and stored at −15° in small aliquots. ε_{537} of 6.05×10^4 was used for assaying concn [8].

Amaranthin was prepared from half-seedlings [3] of *A. tricolor* which had been harvested and aged (1.5 hr at 40°, then 1.5 hr at 25°), then incubated for 24 hr in 5 μM benzyladenine in 10 mM Na_2HPO_4–KH_2PO_4, pH 6.8. The plant material from 1 g seeds was homogenized in 60 ml 3.33 mM HOAc, centrifuged at 48 000 *g* for 60 min and the supernatant freeze-dried. The solid material was resuspended in 2.5 ml 0.1 M citrate buffer, pH 3.4, and applied to a column of Sephadex G-25 as described for betanin. ε_{537} of 5.66×10^4 was used for assaying concn [16].

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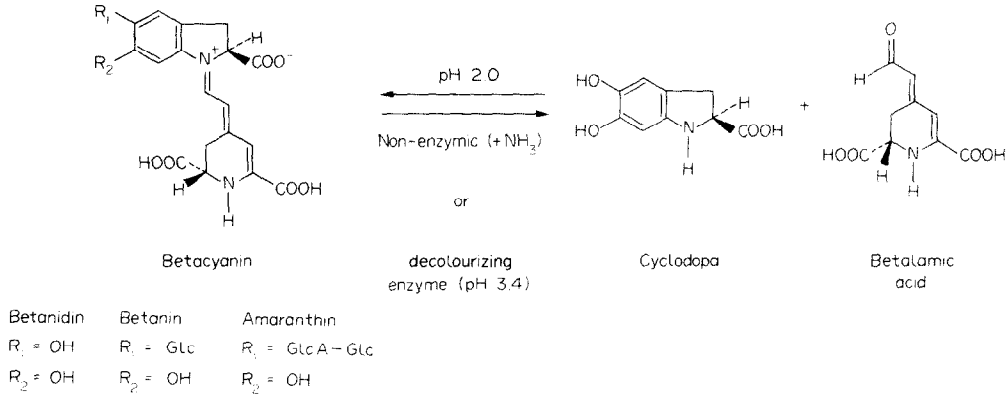

Fig. 2. Enzymic and spontaneous catabolism of betacyanin.

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