Phytochemistry, 1975, Vol. 14, pp. 2135–2139. Pergamon Press. Printed in England.

2,3-DIHYDROXYBENZOATE 2,3-OXYGENASE FROM THE CHLOROPLAST FRACTION OF *TECOMA STANS*

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(*Received 5 December 1974*)

Key Word Index—*Tecoma stans*; Bignoniaceae; 2,3-dihydroxybenzoate 2,3-oxygenase; chloroplast; enzyme properties; metal ion requirement; polyphenol oxidase.

Abstract—2,3-Dihydroxybenzoate-2,3-oxygenase is mainly localized in the soluble and the chloroplast fractions of *Tecoma* leaves. It is associated with the lamellar structure of the chloroplast fraction. The chloroplast enzyme has properties similar to those of the soluble enzyme, but it has a longer half-life and is more stable to dialysis than the soluble enzyme. It is inhibited by sulfhydryl reagents and the inhibition is reversed by the addition of reduced glutathione. The chloroplast enzyme is insensitive to iron-chelating agents. The enzyme loses activity on dialysis against copper-chelating agents and the activity is completely recovered on the addition of copper; addition of iron does not restore the activity. Polyphenol oxidase is probably present only in the active form in the *Tecoma* chloroplast but it is not involved in the intradiol cleavage of 2,3-dihydroxybenzoic acid.

INTRODUCTION

In an earlier communication [1], we reported the oxidation of 2,3-dihydroxybenzoic acid (2,3-DHBA) by the β-ketoadipate pathway in the cell-free extract of leaves of *Tecoma stans*. The first enzyme of the pathway, namely 2,3-dihydroxybenzoate-2,3-oxygenase has been partially purified from the supernatant fraction of *Tecoma* leaves [2]. It was of interest to explore the intracellular distribution of this enzyme. Although the bulk of the enzyme activity was present in the soluble fraction, 25% of the total activity was associated with the chloroplast fraction. In the present communication, we report the properties of the enzyme associated with the chloroplast fraction and compare it with those of the soluble fraction.

RESULTS AND DISCUSSION

Distribution of the enzyme. Maximum activity is localized in the soluble fraction (154 units/g tissue) while the chloroplast fraction exhibits about 30% of the supernatant activity (47 units/g tissue). This activity could not be leached out by washing the chloroplast fraction. Mitochondrial and microsomal fractions contained negligible activity (10 and 4 units/g tissue resp.).

The isolated products of the reactions catalysed by the chloroplast and the soluble enzyme were the same and identified as 2,6-dioxa-3,7-dioxo*bicyclo*(3:3:0)octane-8-carboxylic acid [1] by co-chromatography in different solvent systems, and UV spectra in neutral, acidic and alkaline conditions.

The chloroplast enzyme is more stable than the soluble enzyme—their half-lives being 8–9 and 4–5 hr respectively. Attempts to solubilize the chloroplast enzyme by organic solvents and non-ionic detergents resulted in complete loss of activity. Even a milder treatment by sonication in hypotonic solution inactivated the enzyme. The sub-chloroplast particles prepared by Tris–HCl and EDTA treatments did not show any activity, but when supplemented with copper, 50 and 100% of the activity could be recovered (Table 1). The properties of the enzyme reported in this paper were therefore studied with the chloroplast fraction as such.

Table 1. 2,3-Dihydroxybenzoate-2,3-oxygenase associated with subchloroplast particles

Treatment	Sp act (units/mg protein)	Protein concn (mg/ml)
Chloroplast fraction	15·5	5·5
Tris–HCl	nd	3·4
+ Cu^{2+} (5×10^{-4} M)	7·1	
EDTA	nd	3·8
+ Cu^{2+} (5×10^{-4} M)	16·7	

The chloroplast fraction was subjected to the Tris–HCl and EDTA treatments as described in the text. The activity of the particles was assayed under standard conditions.
nd = Not detectable.

Optimum conditions for 2,3-dihydroxybenzoate-2,3-oxygenase of the chloroplast. The reaction with the chloroplast enzyme was linear for up to 20 min. Maximum enzyme activity was obtained at 30° and 30% of the total activity was observed at a temperature as high as 60°. The chloroplast enzyme had an acidic pH optimum (5·6 in citrate–phosphate buffer, 0·1 M) and no activity was observed at pH values greater than 7. An initial lag in the velocity of the reaction was observed at low concentration of the substrate followed by a sudden increase on raising the substrate concentration. Maximum activity was observed at a substrate concentration of 8×10^{-4} M.

Properties of chloroplast 2,3-dihydroxybenzoate-2,3-oxygenase. The particulate enzyme is highly susceptible to inhibition by the sulfhydryl reagents as shown in Table 2. At $3{\cdot}3 \times 10^{-4}$ M concentration, *p*CMB caused 82% inhibition of the enzyme but unlike the supernatant enzyme [2], the addition of reduced glutathione reversed the inhibition (Table 2), thus suggesting the possibility that -SH groups are involved in the reaction.

The effect of various metal ions on the chloroplast enzyme are summarized in Table 3. Metal ions like Cd^{2+}, Co^{2+}, Mg^{2+}, Hg^{2+} and Ag^{+} were potent inhibitors of the enzyme activity whereas Mn^{2+} and Zn^{2+} ions were weak inhibitors. Fe^{2+} and Fe^{3+} at 10^{-3} M caused an inhibition of 20 and 40% respectively while Ca^{2+} at the same concentration did not show any inhibitory effect. Cu^{2+} ions caused a 15% inhibition of the activity at 10^{-3} M concentration but at 10^{-5} M and 10^{-4} M concentrations respectively, Cu^{2+} and Mn^{2+} had a slight stimulatory effect on the enzyme activity. The bound and the soluble enzymes differed markedly in their response towards various metal ions (Table 3).

Table 2. Effect of sulfhydryl reagents on chloroplastic 2,3-dihydroxybenzoate-2,3-oxygenase

Supplement	Concentration (M)	Inhibition (%)
None	—	0
GSH	2×10^{-3}	1·3
*p*CMB	10^{-5}	19·4
	10^{-4}	49·3
	$3{\cdot}3 \times 10^{-4}$	81·7
*p*CMB	$3{\cdot}3 \times 10^{-4}$	
+ GSH	2×10^{-3}	7·0
Iodoacetate	10^{-5}	55·0
	10^{-3}	100·0
N-ethylmaleimide	10^{-5}	58·4
	10^{-3}	100·0

The reaction was measured under the standard assay conditions. The chloroplast fraction was preincubated with the sulfhydryl reagents and the reaction was started by the addition of the substrate. GSH was added after the addition of substrate.

Azide, cyanide, semicarbazide and sulphide at 10^{-3} M concentration completely inhibited the chloroplast enzyme. Fluoride and arsenite at 10^{-3} M caused 38 and 62% inhibition respectively. Azide, cyanide and semicarbazide at 10^{-5} M concentration inhibited the enzyme to the extent of 59, 75 and 72% respectively.

Table 3. Effect of various metal ions (10^{-3} M) on the 2,3-dihydroxybenzoate-2,3-oxygenase of chloroplast

Supplement	% Activity	
	Chloroplast	Soluble*
Nil	100	100
Cd^{2+}	0	100
Co^{2+}	21	10
Mg^{2+}	42	44
Hg^{2+}	35	6
Ag^{+}	37	53
Mn^{2+}	72	106
Mn^{2+} (10^{-4} M)	110	106
Zn^{2+}	78	116
Fe^{2+}	78	31
Fe^{3+}	81	37
Cu^{2+}	85	116
Cu^{2+} (10^{-5} M)	110	112

* Figures reproduced from Ref. [2].
The chloroplast fraction was preincubated with different metal ions for 5 min and the reaction was started by the addition of substrate. Activity was determined by the standard procedure.

Table 4. Effect of metal chelating agents on 2,3-dihydroxybenzoate-2,3-oxygenase of chloroplast fraction

Supplement	Concentration (M)	% Inhibition
Experiment a		
1. None	—	0
2. α,α′-dipyridyl	10^{-5}	8
	10^{-3}	32
3. *o*-Phenanthroline	10^{-5}	6
	10^{-3}	39
4. Bathophenanthroline	10^{-5}	0
	10^{-3}	20
5. 8-Hydroxyquinoline	10^{-5}	28
	10^{-3}	59
6. Neocuproine	10^{-5}	81
	10^{-3}	100
7. Diethyldithiocarbamate	10^{-5}	64
	10^{-3}	100
8. Salicylaldoxime	10^{-5}	40
	10^{-3}	81
Experiment b		
1. Neocuproine	10^{-5}	81
+ Cu^{2+}	10^{-4}	14
+ Cu^{2+}	10^{-3}	14
2. Diethyldithiocarbamate	10^{-5}	61
+ Cu^{2+}	10^{-4}	20
+ Cu^{2+}	10^{-3}	6
3. Salicylaldoxime	10^{-5}	54
+ Cu^{2+}	10^{-4}	30
+ Cu^{2+}	10^{-3}	5

The chloroplast fraction was preincubated with the metal-chelating agents for 5 min and the reaction was started by the addition of substrate. The addition of Cu^{2+} ions was made at zero time of the start of reaction. The activity was assayed under the standard conditions.

Table 5. Effect of dialysis of chloroplast fraction against copper-chelating agents and addition of Cu^{2+} ions to the dialysed preparation

Treatment	% Activity
1. Control	100
2. Dialysis against cyanide	0
+ Cu^{2+} (10^{-3} M)	127
3. Dialysis against diethyldithiocarbamate	0
+ Cu^{2+} (10^{-3} M)	113
+ Fe^{2+} (10^{-3} M)	25
+ Fe^{2+} (5×10^{-3} M)	28
+ Fe^{3+} (10^{-3} M)	18
+ Fe^{3+} (5×10^{-3} M)	22
+ Cu^{2+} (8×10^{-5} M)	54
+ Cu^{2+} (8×10^{-5} M) + Fe^{2+} (10^{-3} M)	52
+ Cu^{2+} (8×10^{-5} M) + Fe^{3+} (10^{-3} M)	56
4. Dialysis against salicylaldoxime	36
+ Cu^{2+} (10^{-3} M)	109
5. Dialysis against neocuproine	22
+ Cu^{2+} (10^{-3} M)	85

5 ml of chloroplast fraction was dialysed against 200 ml of various copper complexing agents at 10^{-3} M concn for 3–4 hr at 0–5°. The excess of the reagents in the dialysed fractions was removed by dialysis against 1 l. of soln A (4 intermittent changes) for 4–5 hr. The preparation was incubated with various metal ions for 5 min at 30° and the reaction was started by the addition of substrate. The activity was assayed under the standard conditions.

Since it is well established that all the microbial dioxygenases involved in the ring fission of aromatic compounds require iron for their activity [3], the effect of specific iron-chelating agents on the chloroplast enzyme was tested and the results are given in Table 4.

The results suggest the possibility of copper being the cofactor for the chloroplast enzyme also. This was further confirmed by the fact that the inhibition by copper-chelating agents could be overcome by the addition of Cu^{2+} ions (Table 4).

Unlike the soluble enzyme [2], the chloroplast enzyme was stable to dialysis and further studies were carried out with the dialysed preparation to show the involvement of copper in the enzyme activity. There was a 70–100% loss of enzyme activity when the chloroplast preparation was dialysed against the copper-chelating agents and the activity was completely restored by the addition of Cu^{2+} ions (Table 5). Addition of Fe^{2+} and Fe^{3+} ions did not restore the activity (Table 5). The observation that iron did not cause a further increase in activity in a system where the dialysed preparation of the chloroplast fraction was partially activated by Cu^{2+} ions suggests that it is only copper (and not iron) which is needed for the ring cleavage of 2,3-DHBA by the chloroplast fraction. Similar conclusions were also drawn from various lines of evidence with the soluble enzyme.

Effect of polyphenol oxidase on the ring cleavage of 2,3-*DHBA*. Polyphenol oxidase—a cuproprotein—has been reported from the chloroplast of a number of plants [4–8]. It is present either in active or latent form and its conversion from the latent to active form has been achieved by detergents [7,9], denaturing agents [8], Triton X-100 [10], etc. Tolbert [11] has reported that the activation of latent polyphenol oxidase of spinach chloroplast can be achieved by aging the plant tissue and the chloroplast, freezing and thawing of chloroplast and treating the chloroplast with trypsin and autocatalysed trypsin.

Confirmation that the oxidation of 2,3-DHBA by the chloroplast enzyme of *Tecoma* is not a side reaction of polyphenol oxidase was obtained by the following experiments. Partially purified potato polyphenol oxidase was not active against 2,3-DHBA. Moreover, no product corresponding to 2,6-dioxa-3,7-dioxo*bicyclo*(3:3:0)octane-8-carboxylic acid was detected by chromatography from the large scale reaction mixture of 2,3-DHBA and potato polyphenol oxidase. Though the chloroplast fractions of *Tecoma* contains polyphenol oxidase activity (with catechol as the substrate), it could not utilise 2,3-DHBA as its substrate. The 2,3-dihydroxybenzoate-2,3-oxygenase activity of *Tecoma* chloroplast was lost when the chloroplast fraction was frozen and thawed. When *Tecoma* leaves were stored at 4° and −20° and the chloroplast was prepared, no ring cleavage activity with 2,3-DHBA was observed. The oxygenase activity was lost when the chloroplasts were treated with sodium dodecylsulphate, Tween-20, Triton X-100 and sodium deoxycholate. The chloroplast enzyme was neither activated nor inhibited by trypsin.

The *Tecoma* enzyme exhibits dramatic variations and photoperiodicity; studies on the mechanisms operative in triggering the enzyme will be an interesting area of future research and further studies on the dioxygenase in a system like the chloroplast will prove to be valuable in exploring the problem of the physiological importance of dioxygenases in the metabolism of aromatic compounds.

EXPERIMENTAL

Subcellular fractionation A slightly modified procedure of Ref [12] was used for the preparation of the subcellular fractions of *Tecoma stans* leaves. Freshly collected tender leaves (10–12 days old. 10 g) were washed and ground to a thin slurry with acid-washed glass-powder in 25 ml soln consisting of 10 mM citrate–Pi buffer (pH 5·6) and 0·35 M NaCl (soln A). The mixture was squeezed through a double layer of cheese cloth and centrifuged for 1 min at 50 *g* to remove the whole cells and cell debris. The chloroplast fraction was prepared by centrifugation of the supernatant obtained from the earlier step at 1000 *g* for 5 min. The pellet obtained was resuspended in 10 ml of soln A and the clumped chloroplasts were removed by centrifugation at 50 *g* for 1 min. The ppt was discarded and the supernatant was centrifuged at 1000 *g* for 5 min to collect the chloroplast fraction. The pellet was washed with 20 ml of soln A × 3 and finally the pellet was taken in 10 ml of soln A and this suspension was designated the chloroplast fraction. The post-chloroplast supernatant was centrifuged at 20000 *g* for 20 min. The residue obtained was washed 2× with soln A and the final residue was taken in 10 ml of soln A. This fraction was designated as the mitochondria. The 20000 *g* supernatant was further centrifuged at 105000 *g* for 60 min. The residue designated as the microsome fraction was taken in 5 ml of soln A. The 105000 *g* supernatant was designated as the soluble fraction.

Enzyme assay. The standard reaction mixture consisted of 0·1 ml of 2 mM of 2,3-DHBA, 1 ml of the fraction to be assayed and 0·6 ml of 0·1 M citrate–Pi buffer (pH 5·6) and H_2O in a total vol of 2 ml. The reaction was started by the addition of substrate and carried out at 30° for 15 min in a shaker. The reaction was terminated by the addition of 0·2 ml of 0·5 N HCl and the mixture was extracted ×3 with a total vol of 9 ml of Et_2O. The Et_2O layers were pooled, evaporated to dryness, residue taken up in 2 ml of H_2O and the amount of 2,3-DHBA estimated colorimetrically by the method of Ref. [13]. A substrate blank served as a control in all the experiments. One unit of activity is defined as the amount of enzyme required to bring about the disappearance of 0·1 nmol of substrate per min at 30° under the standard assay conditions. Sp. act. is expressed as units of enzyme activity per mg of protein. Spectrophotometric assay of the enzyme [2] was not possible due to the presence of large quantities of chlorophyll and other Et_2O soluble compounds in the chloroplast fraction.

Isolation of the reaction product. The standard reaction mixture, scaled up ×20, was incubated under the standard conditions. The reaction was terminated by acidification and the reaction mixture was extracted ×3 with a total vol of 120 ml of Et_2O. The Et_2O layers were pooled, dried and concentrated *in vacuo*. The concentrated extract was subjected to ascending preparative TLC on cellulose. The chromatogram was developed in (C_6H_6–HOAc–H_2O) (2:4:1, upper phase). The product was detected on the chromatogram by its green fluorescence under UV, scraped off, extracted repeatedly with Me_2CO and the extract evaporated to dryness at 55°. The residue was dissolved in a small vol. of Et_2O and the reaction product was crystallized twice from EtOAc–Et_2O mixture (yield 2·7 mg). Other solvent systems employed for PC of the product isolated from reaction mixture of chloroplast fraction and the soluble enzyme were, (1) C_6H_6–MeOH–HOAc (5:5:1) (R_f 0·42); (2) Tertiary amyl alcohol–$CHCl_3$–H_2O–HCO_2H (8:8:8:3) (R_f 0·46) and (3) C_5H_5N–HOAc–H_2O (10:7:3) (R_f 0·77).

Preparation of subchloroplast particles. The chloroplast isolated from 20 g of *Tecoma* leaves was suspended in 10 ml soln A and the subchloroplast particles were prepared by the method of Ref. [14].

Protein estimation. The samples were first processed by the method of Ref. [15] to remove the endogenous phenolics and other alcohol soluble materials and the protein content in the residue was determined by the procedure of Ref. [16].

Preparation and assay of potato polyphenol oxidase. The enzyme was prepared according to the method of Ref. [17]. The enzyme at the DEAE-cellulose stage (40–50× purified) was used in the present studies. The activity was assayed by the method of Ref. [18]. The reaction mixture consisted of 0·4 ml of enzyme soln or chloroplast fraction, 0·2 ml of 10^{-3} M catechol and 0·4 ml of 0·1 M NaPi buffer (pH 6). The polyphenol oxidase activity of potato or *Tecoma* chloroplast fraction against 2,3-DHBA was tested at the final substrate concentration of 10^{-3} M and 10^{-4} M.

Acknowledgements—The authors are grateful to Dr. M. Jamaluddin, Temple University, Philadelphia, for helpful suggestions during the course of this work and the criticism of the manuscript before submission for publication.

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