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PEROXIDASE-MEDIATED HYDROXYLATION OF *p*-HYDROXYPHENYLACETONITRILE, AN INTERMEDIATE IN DHURRIN SYNTHESIS IN SORGHUM

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Key Word Index—*Sorghum bicolor*; Gramineae; *p*-hydroxyphenylacetonitrile; aliphatic hydroxylation; peroxidase.

Abstract—A soluble enzyme from sorghum seedlings mediates the aliphatic hydroxylation of *p*-hydroxyphenylacetonitrile. The reaction was dependent on the presence of oxygen, was stimulated by H_2O_2 and Mn^{2+} and inhibited by CN^-. The enzyme behaved as a classic peroxidase with *o*-dianisidine and NAD(P)H and several methods failed to separate hydroxylase from peroxidase function. Horseradish peroxidase was found to act in the same manner as the sorghum enzyme in these reactions.

INTRODUCTION

Recent *in vivo* feeding experiments and work with cell-free systems has supported the proposed [1] pathway for dhurrin synthesis in sorghum seedlings (Fig. 1). Thus tyrosine (**1**) [2, 3], *p*-hydroxyphenylacetaldoxime (**2**) [4], *p*-hydroxyphenylacetonitrile (**3**) [4] and *p*-hydroxymandelonitrile (**4**) [5] have been shown to be specifically incorporated into the glycoside (**5**). Using microsomal preparations MacFarlane *et al.* [6] have demonstrated the conversion of tyrosine to the cyanohydrin (**4**). Addition of UDP-glucose and a fraction from the high speed supernatant allowed the total synthesis of dhurrin from tyrosine.

Other experiments have shown [7] that a soluble enzyme fraction from sorghum homogenates can catalyse the hydroxylation of the nitrile (**3** → **4**). This paper reports details of this reaction.

* The nature of these reactions will be the subject of a future communication.

RESULTS

Assay system

Cyanohydrins such as **4** break down rapidly in aqueous solution yielding aldehydes and HCN. The progress of hydroxylation of **3** could therefore be monitored by assaying either *p*-hydroxybenzaldehyde or HCN formed. A 1:1 stoichometry was demonstrated for product formation (Table 1) although some enzyme preparations were able to oxidise the aldehyde to other products including *p*-hydroxybenzoic acid.* Proof that the HCN measured was derived solely from the nitrile substrate was gained by incubating *p*-hydroxyphenylacetonitrile-1-[^{14}C]substrate (1.35×10^4 dpm/μmol; conditions as for Table 1) and recovering HCN with the same sp. act.

The progress of hydroxylation was most rapid during the first few minutes of the reaction (Fig. 2) with the slower phase of the reaction remaining linear with time for at least 30 min. More reliable values for initial rates were obtained by monitoring aldehyde formation at 330 nm. Proof that the increase in A at this wavelength was due

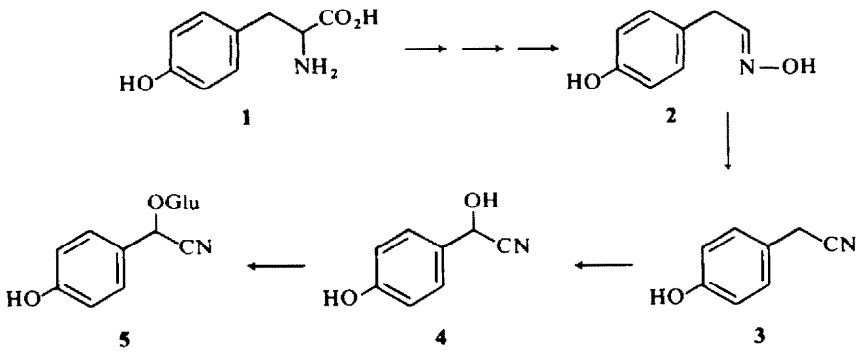

Fig. 1. Proposed pathway for dhurrin synthesis from tyrosine.

Table 1. Isolation of products following hydroxylation of *p*-hydroxyphenylacetonitrile

Product isolated (nmol)	Incubation time (min) 0	3	10
HCN	0	125	133
p-Hydroxybenzaldehyde	0	129	137

Conditions in experimental using Pi buffer, 3 μmol/ml *p*-hydroxyphenylacetonitrile and 27 μg/ml enzyme protein (40–60% AS fraction). Controls with boiled enzyme produced no HCN or aldehyde.

to *p*-hydroxybenzaldehyde was gained by isolating the compound from assay mixtures and comparison of UV spectra and R_f values on TLC with an authentic sample.

Distribution of enzyme activity

Accurate determination of activity in crude extracts was complicated by the high level (*ca* 7 μmol/ml) of cyanide generated by the breakdown of endogenous dhurrin. Cyanide inhibits the hydroxylation reaction (Table 6) and was not completely removed by prolonged dialysis. However, at least 80% of the total activity was present in the high speed supernatant and most was precipitated in the 50–60% ammonium sulphate (AS) fraction (Table 2). Fractions catalysing the hydroxylation of *p*-hydroxyphenylacetonitrile also exhibit peroxidase activity with σ-dianisidine as substrate (Table 2). The 50–60% AS fraction contains the greatest proportion in each case but lower fractions contain significant amounts of peroxidase activity. When the 75000 *g* supernatant was submitted to DE-32 cellulose chromatography 3 peaks of activity were obtained (Fig. 3), each of which catalysed the hydroxylation and peroxidative reactions. However, the ratio of activities varied between peaks (Table 3). Attempts to alter this ratio by partial heat denaturation were unsuccessful: both forms of activity decreased by *ca* 50% after heating at 50° for 30 min. The pH optima for hydroxylation and peroxidation were 7.5 and 5–5.5 respectively.

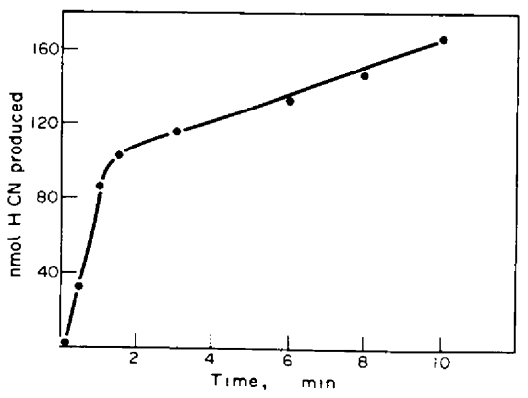

Fig. 2. Progress of hydroxylation of *p*-hydroxyphenylacetonitrile measured by HCN release. Conditions of assay given in Experimental, method (a).

Table 2. Relative activities during ammonium sulphate (AS) fractionation expressed as a percentage of activity in 75000 *g* supernatant

Fraction	Reaction and substrate: Hydroxylation (*p*-hydroxyphenylacetonitrile)	Peroxidation (σ-dianisidine)
75000 *g* Supernatant	100	100
0–40% AS fraction	4	16
40–50% AS fraction	7	32
50–60% AS fraction	86	48
60–100% AS fraction	3	4

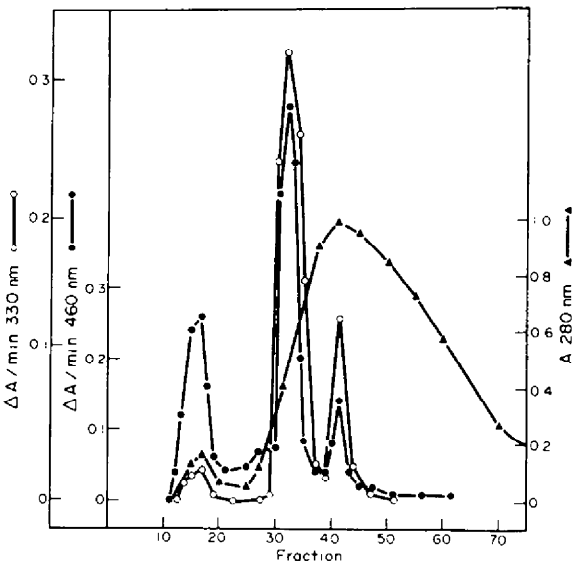

Fig. 3. Elution profile from DE-32 Cellulose column using a linear gradient from 0.01 M to 0.2 M K Pi buffer pH 8. Aliquots from 10 ml fractions were assayed for peroxidase (●- - -●) or hydroxylase (○——○) activity. Tubes 12–19, 30–37 and 40–43 were combined to form fractions A, B and C.

Table 3. Ratio of peroxidase and hydroxylase activities of fractions after DE-32 cellulose chromatography

Enzyme	Peroxidase activity	Hydroxylase activity	Ratio of peroxidase / hydroxylase
75000 *g* Supernatant	14	54	0.26
Fraction A	227	92	2.5
B	34	117	0.29
C	8	72	0.11

Peroxidase activity = change in A (460 nm)/min/mg protein
Hydroxylase activity = nmol *p*-hydroxybenaldehyde formed/min/mg protein.

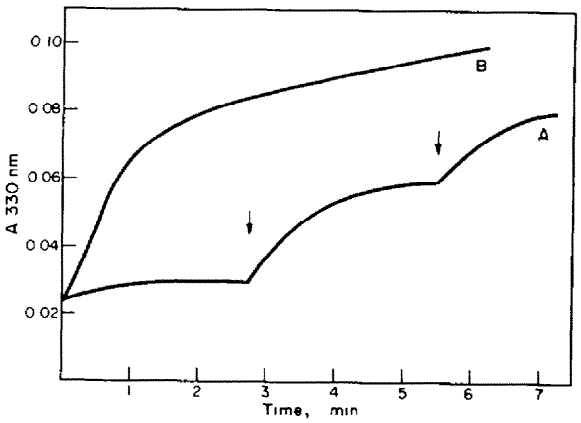

Fig. 4. Oxygen requirement for hydroxylation of *p*-hydroxyphenylacetonitrile. Curve A: standard reaction mixture (see Experimental) in Thunberg cuvette with substrate in side-arm. After evacuation and flushing with argon the components were mixed and incubated in the spectrophotometer at 30°. After some time the cuvette was opened and stirred (arrows). Curve B: same reaction in open cuvette.

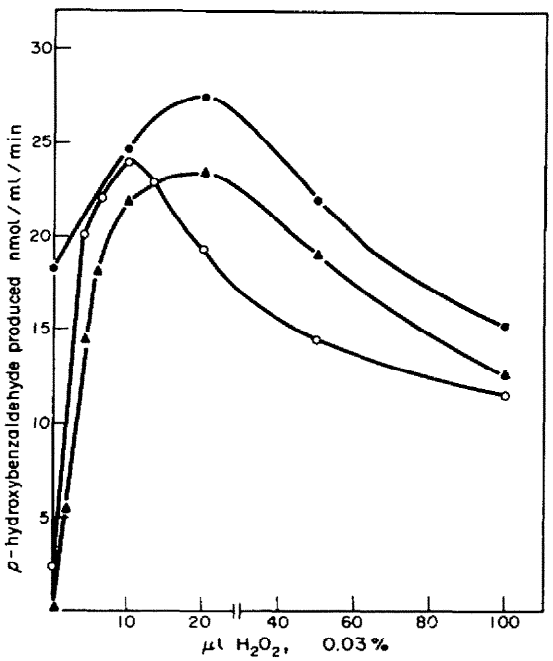

Fig. 5. Stimulation of rate of hydroxylation of *p*-hydroxyphenylacetonitrile by added H_2O_2. Standard spectrophotometric assay with 0.22 mg protein in 0.1 M Pi (●——●), 0.1 M Tris (○——○) and 0.1 M Tricine (▲——▲) buffers.

Although there was no absolute cofactor requirement for the hydroxylation of *p*-hydroxyphenylacetonitrile the addition of Mn^{2+} more than doubled the rate at a level of 50 nmol/ml. No further increase in rate occurred above this concentration. The presence of Fe^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} had no effect on reaction rate. However, it was found necessary for oxygen to be present before the reaction would proceed. No HCN or *p*-hydroxybenzaldehyde could be detected in assay mixtures when reactions were carried out in Thunberg tubes or cuvettes flushed with argon but when air was admitted the reaction began (Fig. 4).

The nature and concentration of the buffer used in the assay had a marked effect on the rate of hydroxylation. Reactions in Pi (Na or K) proceeded faster than in Tris, whilst in Tricine buffer there was no detectable hydroxylation over 5 min (Table 4). Addition of H_2O_2 to assay mixtures eliminated the lag period and substantially increased the initial rate of reaction (Fig. 5). Conversely, however, preincubation of the sorghum enzyme with catalase in Pi buffer failed to inhibit the reaction significantly unless large amounts were used; 750 units were necessary to give a 10% reduction in hydroxylation rate.

Table 4. Initial rates of hydroxylation of *p*-hydroxyphenylacetonitrile. Standard spectrophotometric assay (see Experimental) using 0.28 mg protein from 50 to 60% AS fraction

Buffer (M)		*p*-Hydroxybenzaldehyde formed (nmol/min)	Lag time (sec)
K Pi	0.1	39	—
	0.05	34	—
	0.01	24	10
Tris	0.1	11	36
	0.05	8	45
	0.01	4	140
Tricine	0.1	No reaction over 5 min	

The 50–60% AS fraction from sorghum extracts was further shown to behave as a typical peroxidase in that it exhibited NADH/NADPH oxidase activity (Table 5) in a similar manner to horseradish peroxidase [8]. *p*-Hydroxyphenylacetonitrile was able to substitute for resorcinol in the reaction but lower rates of oxidation were observed.

Horseradish peroxidase was also shown to catalyse the hydroxylation of *p*-hydroxyphenylacetonitrile. Similar rates were observed when either 0.05 mg horseradish peroxidase (72 purpurogallin units/mg) or 0.35 mg sorghum peroxidase (50–60% AS fraction) were used under standard assay conditions. Initial rates showed the same dependence on buffer type and concentration as observed

Table 5. Oxidation of NAD(P)H. The reaction mixture contained 0.3 μmol NAD(P)H, 0.03 μmol $MnCl_2$, 0.25 mg sorghum peroxidase and 0.05 μmol resorcinol or *p*-hydroxyphenylacetonitrile in 3 ml 0.1 M K Pi buffer pH 7.5 at 30°. Mixtures omitting $MnCl_2$ or the phenol showed negligible rates of oxidation

Reaction mixture containing:	NAD(P)H oxidized (nmol/min)
NADH	
resorcinol	19.4
p-hydroxyphenylacetonitrile	5.8
NADPH	
resorcinol	21.3
p-hydroxyphenylacetonitrile	7.3

Table 6. Inhibition of sorghum and horseradish peroxidase by CN^-. Standard assay as in Experimental. Enzyme levels were adjusted to give *ca* equal rates in the absence of inhibitor

Enzyme	CN^- concn (M) to give 50% inhibition of: Peroxidation	Hydroxylation
Horseradish	4.2×10^{-6}	1.9×10^{-5}
Sorghum	6.7×10^{-6}	3.0×10^{-5}

with the sorghum enzyme and there was a similar degree of stimulation of hydroxylation in the presence of added Mn^{2+} or H_2O_2. Both enzymes were inhibited in hydroxylation and peroxidation by similar concentrations of CN^- (Table 6).

DISCUSSION

Preliminary studies on the nature of the enzyme involved in the hydroxylation of *p*-hydroxyphenylacetonitrile indicated that the activity was associated with both the microsomal and high-speed supernatant fractions. Work on the microsomal system has been reported elsewhere [6, 9]; this paper shows the nature of the activity in the soluble fraction.

All available evidence indicates that a peroxidase is responsible for the hydroxylation of the nitrile. This is supported by a comparative study of the catalytic properties of the sorghum enzyme with horseradish peroxidase. Fractions obtained from either AS precipitation or DE-32 cellulose chromatography exhibited both hydroxylase and peroxidase activities and other separation methods employed (gel filtration, cellulose acetate electrophoresis) also failed to achieve separation. Data from heat denaturation experiments also indicate that both forms of activity are contained in the one enzyme. The peaks of activity following DE-32 cellulose chromatography suggest the existence of isozymic forms and in this regard are consistent with the work of Stafford [10] which demonstrated the presence of several peroxidase isozymes in sorghum extracts.

It is of interest that although attempts to separate hydroxylase from peroxidase activity were unsuccessful, separate fractions do show different activity ratios (Fig. 3, Table 3). This result is similar to those obtained by other groups examining the IAA oxidase peroxidase activity ratios of isozymes from a variety of plant sources [11] and can be rationalised in terms of differing substrate specificities shown by the isozymes [12].

The ability of the sorghum extracts to act as an NAD(P)H oxidase is further evidence of the similarity to horseradish peroxidase [8], with *p*-hydroxyphenylacetonitrile showing the characteristics of an oxidogenic phenol in this reaction (Table 5). Under the assay conditions employed (molar ratio of NAD(P)H to nitrile of 6:1) no hydroxylation of the nitrile was evident; however at higher nitrile concentrations both hydroxylation and NAD(P)H oxidation occurred concurrently.

The requirement for oxygen during hydroxylation and the stimulation of the reaction by added H_2O_2 or Mn^{2+} seem typical of the 'oxidase' function of a peroxidase [13]. There is firm evidence for the generation of free radicals during this process [14] which are capable of reducing oxygen to H_2O_2 via the superoxide radical anion [13]. The mechanism proposed for this process requires trace amounts of H_2O_2 to initiate the reaction in an aerobic environment, which implies that the reaction should be prevented or inhibited by prior incubation with catalase. In the present study only partial inhibition of hydroxylation is observed under these conditions and only then with large amounts of catalase. This implies very tight binding within the H_2O_2 peroxidase complex and is supported by previous work where catalase was unable to completely inhibit the oxidase function [15, 16].

The main interest in this work has been the aliphatic nature of the hydroxylation mediated by peroxidase from sorghum and horseradish. This function must now be added to the wide range of reactions catalysed by the enzyme [13, 17, 18]. The stereospecificity of hydroxylation has been examined and compared with that of a second hydroxylase present in sorghum microsomes [19]. The role of both enzymes in the biosynthesis of dhurrin is fully discussed in that paper. It is sufficient to add at this point that evidence indicates the microsomal enzyme to be directly involved in dhurrin synthesis and that aliphatic hydroxylation of the intermediate *p*-hydroxyphenylacetonitrile described here is yet another of the facets exhibited by peroxidase.

EXPERIMENTAL

Sorghum bicolor seed was a generous gift from Arthur Yates & Co. Pty. Ltd., New South Wales. Horseradish peroxidase (Type 1) was purchased from Sigma Chemical Co. and *p*-hydroxyphenylacetonitrile from Aldrich Chemical Co. *p*-Hydroxyphenylacetonitrile-1-[^{14}C] was synthesized as described previously [4]. All other chemicals were reagent grade.

Preparation of enzyme extracts. Seeds of *S. bicolor* were germinated for 3 days at 25° in the dark. The shoots were harvested and either ground in a chilled mortar or homogenized in a blendor with 10% by wt Polyclar AT and two vols of 0.1 M K Pi pH 7.5. The homogenate was strained through muslin, centrifuged at 20000 *g* and the pellet discarded. Following centrifugation at 75000 *g* the supernatant was subjected to AS fractionation. Fractions were dialysed against 0.1 M K Pi pH 7.5. In expts examining the subcellular distribution of the enzyme 0.1 M K Pi pH 7.5/0.3 M sucrose was used as the extracting buffer.

Assays for hydroxylation of p-hydroxyphenylacetonitrile. (a) *Determination of* HCN *release.* Incubations over periods greater than a few min were conducted in 25 ml conical flasks with a centre well containing NaOH (1 N, 0.5 ml). The main body of the flask contained in a 3 ml vol of K Pi (0.1 M, pH 7.5) $MnCl_2$ (0.15 μmol) and enzyme (*ca* 0.5 mg). After 5 min preincubation with shaking at 30° *p*-hydroxyphenylacetonitrile was injected and the flask sealed with a serum cap. Reactions were terminated by the injection of H_2SO_4 (6 N, 0.2 ml) and shaking continued overnight to facilitate HCN transfer to the centre well. The NaOH/NaCN soln was made up to known vol and aliquots analysed for cyanide by the method of ref. [20].

Assays over short periods were carried out in open test tubes and reactions terminated by NaOH addition (5 N, 0.2 ml). Aliquots were analysed directly for HCN and/or *p*-hydroxybenzaldehyde. Controls showed no significant loss of HCN before NaOH addition. (b) *Spectrophotometric determination of p-hydroxybenzaldehyde formation.* Assay mixtures were as described above. Following injection of the nitrile the reaction was followed at 330 nm against a buffer blank using a spectrophotometer equipped with scale expansion. The molar A of *p*-hydroxybenzaldehyde at 330 nm was 1.07×10^4 in buffer pH 7.5 and 2.73×10^4 in NaOH (1 N). (c) *Isolation of p-hydroxybenzaldehyde from incubation mixtures (a) or (b) above.* Incubation mixtures were adjusted to pH 6 and extracted ×3 with Et_2O. The combined extracts were taken to dryness, redissolved in 95% EtOH and aliquots used for PC (Whatman

3 MM; *n*-BuOH–conc NH_3, 4:1) or TLC (Si gel; C_6H_6–EtoAc, 5:1). Spots were detected by UV or spraying with 2,4-dinitrophenylhydrazine (0.1% in 2 N HCl). Isolated and authentic samples of the aldehyde had identical R_f values (0.38 and 0.59 in above systems) and superimposable UV spectra.

Assay of peroxidase activity. Enzyme samples were added to 3 ml of a mixture of *o*-dianisidine (0.25 ml of 1% soln in MeOH) and H_2O_2 (30 ml of 0.003% in 0.01 M K Pi pH 6) in a cuvette at 30° and the increase in A measured at 460 nm.

Assay of NADH/NADPH oxidase activity. The method of ref. [8] was used.

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