Oxidase–Peroxidase Enzymes of *Datura innoxia* OXIDATION OF FORMYLPHENYLACETIC ACID ETHYL ESTER

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An enzyme system from *Datura innoxia* roots oxidizing formylphenylacetic acid ethyl ester was purified 38-fold by conventional methods such as $(\text{NH}_4)_2\text{SO}_4$ fractionation, negative adsorption on alumina C, gel and chromatography on DEAE-cellulose. The purified enzyme was shown to catalyse the stoichiometric oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester and formic acid, utilizing molecular O_2 . Substrate analogues such as phenylacetaldehyde and phenylpyruvate were oxidized at a very low rate, and formylphenylacetoneitrile was an inhibitor of enzyme activity. The enzyme was inhibited by metal-chelating agents, cyanide, thiol compounds and ascorbic acid. This enzyme was identical with an oxidase–peroxidase isoenzyme. Another oxidase–peroxidase isoenzyme which separated on DEAE-chromatography also showed formylphenylacetic acid ethyl ester oxidase activity, albeit to a lesser extent. The properties of the two isoenzymes of the oxidase were compared and shown to differ in their oxidation and peroxidation properties. The oxidation of formylphenylacetic acid ethyl ester was also catalysed by horseradish peroxidase. The *Datura* isoenzymes exhibited typical haemoprotein spectra. The oxidation of formylphenylacetic acid ethyl ester was different from other peroxidase-catalysed reactions in not being activated by either Mn^{2+} or monophenols. The oxidation was inhibited by several mono- and poly-phenols and by catalase. A reaction mechanism for the oxidation is proposed.

Fig. 1. Formulae of key compounds

amine and scopolamine (Gibson & Youngken, 1967). This suggestion is based on incorporation of radioactive isotopes from differentially labelled phenylalanine into tropic acid with either intact plants or root cultures of several *Datura* species such as *Datura stramonium*, *Datura metel* and *Datura innoxia* (Leete, 1962a,b; Gross & Schutte, 1963). The conversion involves the reduction of the aldehyde group of formylphenylacetic acid to the alcohol group of tropic acid, suggesting that the biological reaction may involve an alcohol dehydrogenase and a reduced nicotinamide nucleotide coenzyme, NADH or NADPH. Formylphenylacetic acid, however, has not been chemically synthesized, owing to its instability, and hence its role in the biosynthesis of these alkaloids has not been directly tested.

During our attempts to study the enzymic reduction of a chemically synthesized derivative of formylphenylacetic acid, namely formylphenylacetic acid ethyl ester (Fig. 1a) by *D. innoxia* root extracts in the presence of NADH or NADPH, we found that these coenzymes were rapidly oxidized to NAD^+ or NADP^+ without concomitant formation of tropic acid ethyl ester, the expected reduction product of formylphenylacetic acid ethyl ester. Under the same conditions, a rapid uptake of O_2 from the medium was also observed, and the oxidation of the coenzymes was dependent on the presence of formyl-

Formylphenylacetic acid (Fig. 1a) has been suggested as the immediate precursor of tropic acid (Fig. 1b), the acid moiety of the alkaloids hyoscy-

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phenylacetic acid ethyl ester, O_2 and the root extracts. O_2 uptake was also observed when formylphenylacetic acid ethyl ester alone was incubated with the root extracts in the absence of NADH or NADPH. Thus two reactions were distinguished under the above conditions: (i) enzymic oxidation of formylphenylacetic acid ethyl ester by O_2 , and (ii) the oxidation of NADH by molecular O_2 which was dependent on the presence of formylphenylacetic acid ethyl ester and the root extracts.

This paper describes the characterization of the first reaction, namely the enzymic oxidation of formylphenylacetic acid ethyl ester, and the oxidase-peroxidase nature of the enzyme that catalyses the reaction. The oxidation of NADH by the *Datura* enzyme in the presence of formylphenylacetic acid ethyl ester is the subject matter of the following paper (Kalyanaraman *et al.*, 1975).

Experimental

Materials

Chemicals. Formylphenylacetic acid ethyl ester was prepared by the procedure of Larsson & Tammelin (1961) and the pure fraction distilling at 110°C at 800 Pa (6 mmHg) was collected. The mass spectrum of the compound showed the expected parent peak of mass 192. Formylphenylacetoneitrile (m.p. 158°C) was prepared by the procedure of Ghosh (1916). Benzoylformic acid ethyl ester [distilling at $116\text{--}118^\circ\text{C}/670\text{ Pa}$ (5 mmHg)] (Fig. 1c) and benzoylformic acid (m.p. 58°C) (Fig. 1c) were prepared by the method of Oakwood & Weisgerber (1955). Horseradish peroxidase (type VI, 260 purpurogallin units/mg) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Catalase (from ox liver, 2000 units/mg) was a product of Worthington Biochemicals Corp. (Freehold, N.J., U.S.A.). All other chemicals were commercial samples of high purity. Alumina C_γ gel was prepared by the procedure of Colowick (1955).

Methods

Enzyme assay. The enzymic oxidation of formylphenylacetic acid ethyl ester was followed by incubating the enzyme with $2.5\text{ }\mu\text{mol}$ of formylphenylacetic acid ethyl ester dissolved in $50\text{ }\mu\text{l}$ of ethanol in 2 ml of 0.2 M -sodium phosphate buffer, pH 7.0. O_2 uptake was followed in an Oxygraph (model K; Gilson Medical Electronics, Middleton, Wis., U.S.A.). In all the experiments with the enzyme purified on DEAE-cellulose, 10–20 ng of enzyme protein was used, because the reaction rate was linear in this range. A slow non-enzymic oxidation of the substrate also occurred, and hence a non-enzymic control was routinely run and the net oxidation due to the enzyme was calculated by deducting the non-enzymic rate.

Measurement of benzoylformic acid ethyl ester. Benzoylformic acid ethyl ester was hydrolysed to benzoylformic acid and measured by a colorimetric method using 2,4-dinitrophenylhydrazine, as follows. The enzymic reaction (standard reaction mixture, volume 2 ml) was stopped by the addition of 1 ml of 5% (w/v) NaOH, which also hydrolysed benzoylformic acid ethyl ester to the free acid almost instantaneously. After 10 min at room temperature (25°C) the reaction mixture was acidified by the addition of 1 ml of 10% (w/v) orthophosphoric acid and extracted with $3\times 4\text{ ml}$ of benzene to remove the unchanged substrate. Benzoylformic acid was measured, in a 1 ml portion of the aqueous layer (containing $5\text{--}40\text{ }\mu\text{g}$ of the compound) by the addition of 0.25 ml of a 0.2% solution of 2,4-dinitrophenylhydrazine in 2 M -HCl and heating the mixture at 70°C for 10 min. After cooling, 3 ml of 10% NaOH was added and the E_{470} of the solution was measured in a Beckman DU spectrophotometer.

Measurement of formic acid. Formic acid was measured by the method of Grant (1948), wherein formic acid was first reduced to formaldehyde with magnesium metal and HCl and was then colorimetrically measured by using chromotropic acid (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) reagent. The standard reaction mixture, after the indicated period of incubation, was chilled in ice and simultaneously extracted with $3\times 4\text{ ml}$ of benzene to remove all unchanged substrate and benzoylformic acid ethyl ester. Formic acid was then measured in a 0.5 ml portion of the aqueous layer. Since phosphate ions were present in the standard enzyme assay reaction mixture, which lowered the colour values, the standard curve for formic acid was also prepared in the presence of 0.02 M -sodium phosphate.

Assay of peroxidase. Peroxidase was assayed by using *o*-dianisidine and H_2O_2 (Worthington Biochemical Corp., 1961).

Measurement of protein. Protein was estimated by the method of Lowry *et al.* (1951), with dry bovine serum albumin (Sigma) as the standard.

Results

Extraction and purification of the enzyme

The roots of 45–100-day-old *D. innoxia* plants, raised in the Institute garden, were used for the extraction of the enzyme. The roots were washed with water, blotted dry and used immediately. All operations were carried out at $0\text{--}4^\circ\text{C}$.

Step I. Roots (100 g) were homogenized in a Waring Blender for 2 min with 200 ml of 2 mM -sodium phosphate buffer, pH 7.0, containing 1 mM -EDTA. The homogenate was filtered through folds of cheesecloth and centrifuged at 27000 g for 10 min in a

Sorvall RC-2B centrifuge. The high-speed supernatant was used for further purification of the enzyme.

Step II. The supernatant from Step I was treated with packed alumina C_γ gel (1.53 g dry wt.), stirred for 30 min and centrifuged at 27000g for 10 min. The residue was discarded. The supernatant (C_γ I fraction) was used for further purification of the enzyme.

Step III. The C_γ I fraction was fractionated with $(NH_4)_2SO_4$. The precipitate appearing between 40 and 80% saturation of $(NH_4)_2SO_4$ contained most of the activity. The precipitate was dissolved in 5 ml of 2 mM-sodium phosphate buffer, pH 7.0, and dialysed against 2 litres of the same buffer for 15 h. The contents of the dialysis bag were centrifuged to remove any undissolved materials.

Step IV. The enzyme from Step III was treated with packed alumina C_γ gel (153 mg dry wt.), stirred for 15 min and centrifuged at 27000g for 10 min. The supernatant had almost all the enzyme activity and was designated C_γ II fraction.

Step V. The C_γ II fraction (25 mg of protein) was loaded on a column of DEAE-cellulose (20 cm $\times$ 1.2 cm), previously equilibrated with 2 mM-sodium phosphate buffer, pH 7.0. After washing with 20 ml of the same buffer the enzyme was eluted with 600 ml of the buffer containing a linear gradient of NaCl (0–0.2 M). Fractions (3 ml) were collected and the formylphenylacetic acid ethyl ester oxidase activity and the protein content of the fractions were determined. A typical elution pattern is given in Fig. 2. Two peaks of oxidase activity, peaks I and II, were eluted from the column. In most experiments described in this paper peak-I enzyme alone was used, which was about 38-fold purified over the crude supernatant and had 25% of the total activity of the Step-I preparation. The purification of formylphenylacetic acid ethyl ester oxidase through various steps is given in Table 1.

Identification of the reaction products

Because of an initial observation that a decrease in the u.v. absorption of the substrate occurred at its λ_{max} , 273 nm, the absorption spectrum of the reaction mixture was scanned between 220 and 340 nm in a Cary model 14 spectrophotometer, at various time-intervals after the addition of the enzyme to the reaction mixture. The scans are shown in Fig. 3(b). During the reaction the absorbance at 273 nm decreased and a new peak appeared initially around 263 nm, which gradually shifted with time to 252 nm, with a concomitant increase in absorption at this wavelength. In the control, which consisted of an identical reaction mixture but without the enzyme, there was a slow decrease in absorption at 273 nm with no simultaneous increase at 263 or 252 nm (Fig. 3a).

A comparison of the u.v. spectrum of the final product, with λ_{max} = 252 nm and an ϵ greater than that for formylphenylacetic acid ethyl ester, with the u.v. spectra of several possible oxidative degradation products of formylphenylacetic acid ethyl ester suggested that the enzymic product could be

Fig. 2. DEAE-cellulose chromatography of the formylphenylacetic acid ethyl ester oxidase

—, Oxidase activity (I, isoenzyme I; II, isoenzyme II);
---, protein content. Experimental conditions are described in the text.

Table 1. Purification of formylphenylacetic acid ethyl ester oxidase from *D. innoxia*

The reaction mixture contained in 2 ml of 0.2 M-phosphate buffer, pH 7.0, 2.5 μ mol of substrate and enzyme. Reaction was followed by monitoring O_2 uptake in the Oxygraph. One unit of enzyme activity is defined as that amount of enzyme which causes an uptake of 1 μ mol of O_2 /min.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
I High-speed supernatant	6200	35.5	174	100	—
II C_γ I enzyme	6200	15.5	400	100	2.3
III $(NH_4)_2SO_4$ (40–80%-satd. fraction)	3280	6.0	547	53	3.14
IV C_γ II enzyme	3280	2.27	1440	53	8.27
V DEAE-cellulose chromatography (peak I)	1690	0.26	6500	27	37.4

Fig. 3. Changes in u.v.-absorption spectrum during the enzymic oxidation of formylphenylacetic acid ethyl ester

(a) Control experiment done under conditions identical with those in (b) except that enzyme was omitted from the reaction mixture. (b) Reaction mixture contained $1\text{ }\mu\text{g}$ of isoenzyme I and $0.28\text{ }\mu\text{mol}$ of formylphenylacetic acid ethyl ester in 3 ml of 0.2M-phosphate buffer, pH 7. The spectrum of the reaction mixture was recorded at the following time-intervals against the same buffer: curve 1, 0 min; 2, 16 min; 3, 32 min; 4, 48 min; 5, 64 min; 6, 128 min; 7, 184 min; 8, 244 min; 9, 394 min; ----, after 20 h.

benzoylformic acid (Fig. 1c), which has $\lambda_{\text{max.}} = 252\text{ nm}$ and ϵ 11 100. If the final product was benzoylformic acid it could have been formed by the hydrolysis of an initial product, namely its ethyl ester, which has $\lambda_{\text{max.}} = 263\text{ nm}$ at pH 7.0 in phosphate buffer. The initial formation of benzoylformic acid ethyl ester was suggested by the transient appearance of an absorption peak at 263 nm (Fig. 3b). Benzoylformic acid ethyl ester was found to hydrolyse slowly at pH 7.0 and instantaneously in alkali to benzoylformic acid.

Further evidence for the initial formation of benzoylformic acid ethyl ester was obtained by t.l.c. The product from the standard reaction mixture after 6 min of incubation was extracted with ether and chromatographed on silica-gel-coated t.l.c. plates with authentic benzoylformic acid and its ethyl ester and formylphenylacetic acid ethyl ester as markers and 7% (v/v) methanol in hexane as the developing solvent. The spots were detected by exposure to iodine vapours. Two major spots, one corresponding to the substrate formylphenylacetic acid ethyl ester (R_F 0.5) and the other having the same

R_F (0.82) as the authentic benzoylformic acid ethyl ester, were identified in the reaction mixture.

Additional proof for the initial formation of benzoylformic acid ethyl ester was obtained by its alkaline hydrolysis to benzoylformic acid and converting the latter to its 2,4-dinitrophenylhydrazone. The identity of the hydrazone of the hydrolysed product with the authentic 2,4-dinitrophenylhydrazone of benzoylformic acid was established by its identical t.l.c. behaviour in two solvent systems [butan-1-ol-ethanol-water (4:1:5, by vol.); product R_F = 0.68, reference R_F = 0.67; and butan-1-ol-acetic acid-water (4:1:5, by vol.) organic phase, product R_F = 0.87, reference R_F = 0.87] and melting point (reference, 196°C ; product, $196\text{--}197^\circ\text{C}$; mixed m.p., 196°C ; all uncorrected).

Stoichiometry of the reaction

Establishment of the stoichiometry between O_2 consumed, formylphenylacetic acid ethyl ester oxidized and benzoylformic acid ethyl ester formed was necessary to trace the fate of the formyl group cleaved

during the conversion of formylphenylacetic acid ethyl ester into benzoylformic acid ethyl ester. The disappearance of the substrate and the formation of benzoylformic acid (formed by the alkaline hydrolysis of the product) were followed by taking advantage of the large differences in their molar absorbances at 250 and 275nm in 0.05M-NaOH ($\epsilon_{250} = 6900$; $\epsilon_{275} = 14410$ for formylphenylacetic acid ethyl ester, and $\epsilon_{250} = 11020$, $\epsilon_{275} = 2100$ for benzoylformic acid). A clear stoichiometry between substrate oxidized and product (benzoylformic acid) formed was established as the ratios of mol of substrate utilized/mol of benzoylformic acid formed were 0.82, 0.94 and 0.96 after 10, 20 and 30min of reaction.

In a parallel experiment benzoylformic acid ethyl ester formation and O_2 uptake were measured simultaneously at two different formylphenylacetic acid ethyl ester concentrations (0.33 and 0.66 $\mu\text{mol/ml}$). The ratios calculated (1.08 and 0.98 respectively) indicated a mol/mol stoichiometry between O_2 consumed and benzoylformic acid ethyl ester formed during the reaction. The results obtained permit the overall reaction to be written as follows:

That formic acid was indeed the other product of the reaction was established by reducing it to formaldehyde with magnesium metal and HCl followed by the colorimetric reaction with the chromotropic acid reagent. Quantitative determination of benzoylformic acid ethyl ester and formic acid formed during the reaction by using two different concentrations of formylphenylacetic acid ethyl ester (1 and 2 $\mu\text{mol/ml}$) indicated that they were formed in equimolar amounts (1.12 and 1.07 mol/mol of substrate respectively).

Time-course of the reaction

The oxidation of formylphenylacetic acid ethyl ester in the presence and absence of the enzyme under standard assay conditions is shown in Fig. 4. The oxidation of formylphenylacetic acid ethyl ester proceeded linearly with time without any lag phase. The rate of the non-enzymic reaction was generally about 5% of the enzymic rate.

Effect of pH on enzyme activity

Fig. 5 represents the effect of pH on enzyme activity. The enzyme functioned optimally at pH 7.0 in phosphate buffer (0.2M) and at pH 7.5 in sodium citrate (0.1M)-sodium phosphate (0.2M) buffer. The enzyme was also stable over a wide range of pH and lost only 30% of activity when exposed to pH 2.0 or 10.6 for 10min.

Effect of formylphenylacetic acid ethyl ester concentration

The effect of substrate concentration on enzyme activity is shown in Fig. 6. The apparent K_m for formylphenylacetic acid ethyl ester was 4.3mM. Standard assay mixtures, however, contained less than saturating concentration of the substrate, owing to its limited solubility.

Fig. 4. Time-course of formylphenylacetic acid ethyl ester oxidation

For details see the text.

Fig. 5. Effect of pH on formylphenylacetic acid ethyl ester oxidation

Reaction mixture (2ml) contained enzyme, 0.2M solution of the appropriate buffer and 2.5 μmol of formylphenylacetic acid ethyl ester. Δ , Sodium citrate-sodium phosphate buffer; $\bullet$, Sodium phosphate buffer; $\circ$, Tris-HCl buffer.

Fig. 6. Effect of formylphenylacetic acid ethyl ester concentration on formylphenylacetic acid ethyl ester oxidase activity

A plot of $1/v$ versus $1/[S]$ is shown. Inset: plot of substrate (formylphenylacetic acid ethyl ester) concentration $[S]$ versus velocity (O_2 uptake/min). Reaction mixture consisted of enzyme and various concentrations of formylphenylacetic acid ethyl ester in 0.2M-phosphate buffer, pH7.0.

Table 2. Substrate specificity of formylphenylacetic acid ethyl ester oxidase

The reaction mixture contained 2.5 μ mol of the specified compound and 2 μ g of the enzyme in 2ml of 0.2M-phosphate buffer, pH7.0. When formylphenylacetic acid ethyl ester was present only 20 ng of the enzyme was used.

Compound	Rate of oxidation (μ mol of O_2 utilized/ min per mg of protein)
Formylphenylacetic acid ethyl ester	4500
Phenylacetaldehyde	6.9
Formylphenylacetoneitrile	0.9
Phenylpyruvic acid	0.5
Ethyl hydrogen phenylmalonate	0
Phenylmalonic acid	0
Atropic acid	0
Ethyl tropate	0
Formylphenylacetic acid ethyl ester + formylphenylacetoneitrile (0.025 μ mol)	3150
Formylphenylacetic acid ethyl ester + formylphenylacetoneitrile (0.25 μ mol)	1450

Substrate specificity

A number of compounds with structural similarity to formylphenylacetic acid ethyl ester were tested as

possible substrates for the oxidative reaction catalysed by the enzyme (Table 2). The oxidation of formylphenylacetic acid ethyl ester was much faster than that of any other compound tested. Only phenylacetaldehyde and to a much lesser extent phenylpyruvic acid and formylphenylacetoneitrile were oxidized. Of these compounds formylphenylacetoneitrile was inhibitory to formylphenylacetic acid ethyl ester oxidation; 70% inhibition was observed when its concentration was only one-tenth the concentration of formylphenylacetic acid ethyl ester in the reaction mixture.

General properties

Mg^{2+} , Zn^{2+} and Co^{2+} at 0.5 mM concentration had no effect on enzyme activity. Mn^{2+} at the same concentration had either no effect or showed a marginal stimulation (about 10%). Cu^{2+} was a potent inhibitor of enzyme activity, an inhibition of 60% being observed at 0.01 M concentration.

Several metal-binding compounds were found to have profound inhibitory effects on the enzyme activity. Diethyl dithiocarbamate and cyanide were the most effective chelating agents, giving 100% inhibition of the enzymic reaction at 0.01 mM-concentration.

The enzyme was not inhibited by either *p*-hydroxy-mercuribenzoate or *N*-ethylmaleimide up to 1 mM concentration.

The enzyme was inhibited by thiol compounds, such as 2-mercaptoethanol, cysteine, GSH and dithioerythritol, cysteine being the most inhibitory, showing 100% inhibition at 0.1 mM concentration. Ascorbic acid and dehydroascorbic acid were also inhibitors of the enzyme.

The properties of the enzyme, particularly the inhibition by KCN, Cu^{2+} , ascorbic acid and thiol compounds, indicated that the enzyme may be an oxidase-peroxidase-type enzyme (Kenten, 1953; Conn & Seki, 1957). When the purified enzyme preparation was tested for peroxidative activity on *o*-dianisidine, it was found to be positive. To examine whether the 'peroxidase' activity is due to the intrinsic nature of the formylphenylacetic acid ethyl ester oxidase itself or to a contaminating protein in the purified preparation, a comparison of 'peroxidase' and 'formylphenylacetic acid ethyl ester oxidase' activities was made.

Enrichment of peroxidase activity, as tested by *o*-dianisidine peroxidation, and of formylphenylacetic acid ethyl ester oxidase activity during the first four steps of purification is given in Table 3. Both the activities are purified during various stages of purification, though somewhat disproportionately, the oxidase activity being purified to a slightly greater extent than peroxidase activity. However, in Step V,

Table 3. Comparison of formylphenylacetic acid ethyl ester oxidase and peroxidase activities during purification of the enzyme from *D. innoxia*

The enzyme was assayed for the two activities under standard assay conditions described under 'Methods'.

Purification step	Sp. activity of formylphenylacetic acid ethyl ester oxidase (μmol of O_2 utilized/min per mg of protein)	Sp. activity of peroxidase (μmol of H_2O_2 decomposed/min per mg of protein)	Ratio
	(a)	(b)	a/b
I Crude high-speed supernatant	170	33.5	5.07
II Negative adsorption on C_7 gel	310	55.4	5.61
III $(\text{NH}_4)_2\text{SO}_4$ (40–80%-satd. fraction)	561	76.4	7.34
IV Second negative adsorption on C_7 gel	1360	200.7	6.77

Fig. 7. Chromatographic distribution of formylphenylacetic acid ethyl ester oxidase and peroxidase activities on DEAE-cellulose

●, Formylphenylacetic acid ethyl ester oxidation activity; ○, *o*-dianisidine peroxidation activity. Experimental conditions are described in the text.

namely chromatography on DEAE-cellulose (Fig. 7), two coincident peaks (I and II) having oxidative and peroxidative activities were eluted, clearly indicating that the same protein fractions catalysed both these reactions. The distribution of the two activities in two peaks also indicates that two isoenzymes are being separated in this step, a view consistent with the known isoenzymic nature of most plant peroxidases (Shannon, 1968). The pooled fractions of peaks I and II are hereafter referred to as isoenzymes

I and II. Differences in the ratio of oxidase to peroxidase activities of the two peaks is apparent in Fig. 7, isoenzyme I having a greater oxidase/peroxidase ratio than isoenzyme II. Both isoenzymes I and II exhibited the typical absorption spectrum (Fig. 8) of a haemoprotein, with sharp absorption maxima at 398 nm for isoenzyme I and 401 nm for isoenzyme II. The absorption maxima shifted to 435 and 437 nm respectively, when the enzymes were reduced with sodium dithionite.

Fig. 8. Absorption spectra of the oxidized and reduced forms of formylphenylacetic acid ethyl ester oxidase isoenzyme I and II

Reduction was carried out with 1 mg of sodium dithionite added to 3 ml of enzyme solution and the spectrum immediately recorded.

Table 4. Comparison of the properties of isoenzymes I and II with horseradish peroxidase

The formylphenylacetic acid ethyl ester oxidase and peroxidase activities were measured by standard procedures, described in the text, and are expressed as μmol of substrate oxidized/min per mg of protein.

Property	Isoenzyme I	Isoenzyme II	Horseradish peroxidase
E_{403}/E_{280}	0.33	0.51	3.0
Formylphenylacetic acid ethyl ester oxidase activity	5950	2700	3200
Peroxidase activity	410	495	1430

Comparison of E_{403}/E_{280} , oxidative and peroxidative properties of isoenzyme I, isoenzyme II and horseradish peroxidase

A comparison of the properties of the above three enzymes is given in Table 4. The ratio E_{403}/E_{280} , an index of the haem content per unit protein, is higher for isoenzyme II (0.51) than for I (0.33). However, both these ratios were much lower than that (3.0) for the purified horseradish peroxidase.

Isoenzyme I had a greater formylphenylacetic acid ethyl ester oxidase activity (2.2-fold) than isoenzyme II, though both had almost the same peroxidative activity. Purified horseradish peroxidase, which had 3.5 times the peroxidase activity of isoenzyme I, had only 53% of the formylphenylacetic acid ethyl ester oxidase activity of this isoenzyme.

Table 5. Substrate specificity of formylphenylacetic acid ethyl ester oxidase in the presence and absence of Mn^{2+}

The reaction mixture contained 2.5 μmol of the compound being tested as substrate, with or without 0.25 mM- Mn^{2+} and a suitable concentration of enzyme in 2 ml of 0.2M-phosphate buffer, pH 7.0.

Compound	Amount of enzyme used (μg)	O ₂ utilized ($\mu\text{mol}/\text{min}$ per mg of protein)	
		- Mn^{2+}	+ Mn^{2+}
Formylphenylacetic acid ethyl ester	0.02	4500	4500
Phenylacetaldehyde	2	6.9	16
Phenylpyruvic acid	2	0.5	5.7
Formylphenylacetoneitrile	2	0.9	1.5

Oxidation of substrates in the presence of Mn^{2+}

Mn^{2+} is required as a cofactor in the horseradish peroxidase-catalysed oxidation of several substrates such as phenylacetaldehyde (Kenten, 1953), phenylpyruvic acid (Conn & Seki, 1957) and oxaloacetic acid (Kay *et al.*, 1967). Hence the oxidative ability of formylphenylacetic acid ethyl ester oxidase isoenzyme I in the presence and absence of Mn^{2+} was tested, and the results are presented in Table 5. Although Mn^{2+} stimulated the oxidation of phenylacetaldehyde about 2.3-fold and that of phenylpyruvic acid about 11-fold, it had no significant effect on the oxidation of formylphenylacetic acid ethyl ester. The low rate of oxidation of formylphenylacetoneitrile was marginally stimulated by Mn^{2+} .

Effect of phenols

Monophenols such as cresols are known to promote peroxidase-catalysed oxidation involving molecular O_2 , such as the oxidation of NADH (Akazawa & Conn, 1958), oxaloacetic acid (Kay *et al.*, 1967) and indolylacetic acid (Kenten, 1955a), whereas polyphenols such as catechol inhibit these reactions. Hence the effect of these phenols on the oxidation of formylphenylacetic acid ethyl ester by isoenzyme I was tested. The results presented in Table 6 show that none of the phenols tested promoted the oxidation of formylphenylacetic acid ethyl ester. Indeed the cresols had an inhibitory effect on the reaction, *m*-cresols being about 68% inhibitory at 1 mM concentration. The polyphenol catechol inhibited the reaction by 93% at 0.01 mM concentration.

Effect of catalase

The effect of catalase, an inhibitor of several peroxidase-catalysed oxidations involving molecular

Table 6. *Effect of phenolic compounds on formylphenylacetic acid ethyl ester oxidase activity*

The enzyme (isoenzyme I) was preincubated with the corresponding phenol for 5 min before the substrate was added to initiate the reaction and the enzyme activity was determined under standard assay conditions. All compounds were at a concentration of 1 mM, except for catechol, which was used at 0.01 mM.

Compound	Inhibition (%)
Salicylic acid	0
Phenol	0
<i>o</i> -Cresol	37
<i>m</i> -Cresol	68
<i>p</i> -Cresol	48
Catechol	95
Resorcinol	45

Table 7. *Effect of catalase on formylphenylacetic acid ethyl ester oxidase activity*

The conditions of the assay were the same as described for standard reaction mixtures except that the enzyme was preincubated with catalase at the given concentrations before initiation of the reaction by the addition of formylphenylacetic acid ethyl ester. Non-enzymic reactions were also carried out with and without catalase under similar conditions. Catalase had no effect on the non-enzymic reaction.

Catalase ($\mu\text{g}/2\text{ml}$ of reaction mixture)	Inhibition (%)
0	—
0.5	37
1.25	80
2.5	87
5.0	88
1.25*	90
1.25†	60

* The substrate was preincubated with catalase for 1 min before starting the reaction by the addition of the enzyme.

† Catalase was added to the reaction mixture 75 s after starting the reaction.

O_2 (Kenten, 1953; Conn & Seki, 1957; Akazawa & Conn, 1958), was tested on formylphenylacetic acid ethyl ester oxidation (Table 7). An 87% inhibition occurred at a concentration of 2.5 μg of catalase in the reaction mixture. Inhibition was nearly complete, however, when the substrate instead of the enzyme was preincubated with catalase (1.25 μg). A 60% inhibition occurred when catalase (1.25 μg) was added 75 s after the initiation of the reaction, which was lower than the 80% inhibition obtained when catalase was added at the start of the reaction. Boiled catalase had no effect on enzyme activity. Ad-

Table 8. *Effect of H_2O_2 on formylphenylacetic acid ethyl ester oxidase activity*

The reaction mixture contained 2.5 μmol of formylphenylacetic acid ethyl ester, the specified concentration of H_2O_2 and enzyme in 2 ml of 0.2 M-phosphate buffer, pH 7.0. Benzoylformic acid ethyl ester formed was measured by the spectrophotometric method.

Concn. of H_2O_2 (mM)	Benzoylformic acid ethyl ester formed (nmol/min per ml)	Activation (%)
—	3.05	—
0.1	3.4	11.5
0.01	3.9	28.0
0.001	3.6	18.0

dition of catalase had no effect on the non-enzymic oxidation of formylphenylacetic acid ethyl ester.

Effect of H_2O_2

Pronounced time-lags in the oxidative reactions of peroxidase have been observed during the oxidation of triose reductone (i.e. dienol form) (Yamazaki *et al.*, 1956), dicarboxylic acids (Kenten & Mann, 1953) and indolylpropionic acid and butyric acid (Kenten, 1955b). This lag was abolished by the addition of H_2O_2 (0.01 mM) to the reaction mixture. During indolylacetic acid oxidation, H_2O_2 not only abolished the lag period but also activated O_2 uptake (Kenten, 1955a). It is generally believed that H_2O_2 participates in the generation of free radicals and thereby hastens the onset of the reaction. Hence the effect of H_2O_2 on formylphenylacetic acid ethyl ester oxidation catalysed by the *Datura* enzyme (isoenzyme I) was tested, and the results are presented in Table 8. Formylphenylacetic acid ethyl ester oxidation, which proceeds without any lag phase, was activated maximally to about 28% at 0.01 mM- H_2O_2 .

Discussion

Formylphenylacetic acid ethyl ester, the esterified form of the postulated intermediate formylphenylacetic acid in the biosynthesis of hyoscyamine, has been shown to be rapidly oxidized by the root extracts of *D. innoxia* and the products of the reaction identified as benzoylformic acid ethyl ester and formic acid. The stoichiometry of the reaction catalysed by the purified enzyme indicates that it is a dioxygenase type of reaction. The substrate specificity of the enzyme shows that the enolic forms of substituted phenylacetaldehyde might have a high affinity for the enzyme; formylphenylacetic acid

ethyl ester is a good substrate and formylphenylacetonitrile is a powerful inhibitor of the enzyme. The observed reactions indicate that formylphenylacetic acid and benzoylformic acid are probably important intermediates in the metabolism of phenylalanine, which may have relevance in the synthesis or breakdown of the tropane alkaloids present in this class of plants.

The reaction has a similarity to the oxidation of phenylacetaldehyde resulting in the formation of benzaldehyde and formic acid, catalysed by pea plant extracts (Kenten, 1953). This reaction is also catalysed by horseradish peroxidase. Other similar reactions are the oxidation of phenylpyruvic acid to benzaldehyde by lupin mitochondria (Conn & Seki, 1957) and the oxidation of indolylacetaldehyde to indol 1-3-yl-aldehyde by horseradish peroxidase (Yeh *et al.*, 1970). However, the C_1 fragment released in these reactions was not identified.

The parallel purification of formylphenylacetic acid ethyl ester oxidation and *o*-dianisidine peroxidation activities and their elution pattern on DEAE-cellulose chromatography indicate that *Datura* formylphenylacetic acid ethyl ester oxidase isoenzymes are really isoenzymes of peroxidase. This inference is supported by the typical haem spectra of the isoenzyme fractions and the inhibition of the formylphenylacetic acid ethyl ester oxidase activity by inhibitors of haem peroxidases such as KCN and metal-chelating agents, Cu^{2+} , thiols and ascorbic acid. Purified horseradish peroxidase also catalyses the oxidation of formylphenylacetic acid ethyl ester. Similar to the properties of horseradish peroxidase, formylphenylacetic acid ethyl ester oxidase is also inhibited by catalase and catechol, and slightly activated by the addition of H_2O_2 , although H_2O_2 itself was not detected during formylphenylacetic acid ethyl ester oxidation. However, formylphenylacetic acid ethyl ester oxidation differed from several peroxidase-catalysed oxidations of substrates such as indolylacetic acid (Kenten, 1955a), oxaloacetic acid (Kay *et al.*, 1967), dicarboxylic acids (Kenten & Mann, 1953), naphthaquinone (Klapper & Hackett, 1963), triose reductone (Yamazaki *et al.*, 1956), phenylacetaldehyde (Kenten, 1953) and phenylpyruvic acid (Conn & Seki, 1957), in being unaffected by the addition of Mn^{2+} . Moreover, monophenols inhibited rather than promoted formylphenylacetic acid ethyl ester oxidation. These differences reflect the greater efficiency of formylphenylacetic acid ethyl ester as a substrate compared with other compounds.

The two isoenzymes I and II of formylphenylacetic acid ethyl ester oxidase differ markedly in their relative oxidative and peroxidative properties. Although the physiological role of the widely distributed peroxidase isoenzymes of plants is not known, differences in their ability to oxidize or

peroxidize a variety of substrates have been reported. Shannon *et al.* (1966) have studied extensively the properties of seven horseradish peroxidase isoenzymes which differed markedly in their oxidative (oxidation of oxaloacetic acid) and peroxidative (peroxidation of *o*-dianisidine) properties. These isoenzymes had different electrophoretic and chromatographic properties as well as different affinities for substrates. Similarly the two peroxidase isoenzymes from wheat germ differed in their ability to oxidize indolylacetic acid, in their pH optima, response to added Mn^{2+} and in their spectral properties (Shin & Nakamura, 1962). Peroxidases from turnip (Hosoya, 1960) and potato (Evans, 1970) have also been shown to differ in their electrophoretic and spectral characteristics. Such differences in the oxidative and peroxidative activities suggest differing physiological and biochemical roles for these isoenzymes in the plant.

Three types of oxidative reactions catalysed by peroxidase involving molecular O_2 have been described: (i) reactions where no oxygen atom is incorporated into the organic reaction products, e.g. oxidation of dihydroxyfumaric acid (Swedin & Theorell, 1940), triose reductone (Yamazaki *et al.*, 1956) and NADH (Akazawa & Conn, 1958); (ii) reactions where one oxygen atom is incorporated into the organic reaction product, as with oxaloacetic acid oxidation (Kay *et al.*, 1967) and indolylacetic acid oxidation (Kenten, 1955a); (iii) reactions where both oxygen atoms are incorporated into the organic reaction products, such as the oxidation of phenylacetaldehyde (Kenten, 1953) and oxidation of indolylacetaldehyde (Yeh *et al.*, 1970). Formylphenylacetic acid ethyl ester oxidation resembles type (iii), with an apparent incorporation of both the atoms of oxygen from molecular O_2 into formylphenylacetic acid ethyl ester to yield the final products, benzoylformic acid ethyl ester and formic acid. By analogy with the mechanisms proposed for the initiation and perpetuation of the oxidation of several substrates such as NADH, dihydroxyfumarate, indolylacetic acid by peroxidase utilizing molecular O_2 and involving the formation of substrate free radical, and in certain cases substrate peroxide free radical and substrate peroxide anion (Yamazaki, 1974), the following mechanism (Scheme 1) is proposed for the oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester and formic acid. The sequence of reactions is initiated by a regular peroxidase-type reaction on the enol form of the substrate yielding the substrate free radical (Step 1). The partial enhancement of the rate of the reaction by H_2O_2 and the inhibition of the reaction by catalase suggests the catalytic involvement of H_2O_2 . Reaction of the substrate free radical with molecular O_2 yields the substrate peroxide radical (Step 2). Such a reaction

Scheme 1. *Proposed mechanism of the enzymic oxidation of formylphenylacetic acid ethyl ester to benzoylformic acid ethyl ester and formic acid*

R, Formylphenylacetic acid ethyl ester.

is envisaged during the oxidation of indolylacetic acid (Yamazaki, 1974). The substrate peroxide free radical then dismutates with another substrate molecule (R in Step 3) to yield a further substrate free radical ($\text{R}^\bullet$) and a substrate peroxide anion, which could internally cyclize and cleave to yield the final products (Steps 4 and 5). A similar mechanism of cyclic peroxide formation before cleavage has been postulated during the oxidation of tryptophan to *N*-formylkynurenine by another haem enzyme, tryptophan pyrrolase (Knox & Tokuyama, 1965; discussion by O. Hayaishi). Such a mechanism requires the incorporation of both the atoms of O_2 molecule into the organic products of the reaction, a hypothesis that can be tested by the use of $^{18}\text{O}_2$.

The oxidations of NADH and dihydroxyfumarate by peroxidase and molecular O_2 are inhibited by the enzyme superoxide anion dismutase, which suggests the intermediate formation of superoxide anion during these reactions. On the other hand, the oxidation of indolylacetic acid by peroxidase is not inhibited by the dismutase enzyme (Yamazaki, 1974). This observation has been explained as being due to the intermediate formation of substrate peroxide complex rather than free superoxide anion species. By analogy, the reaction sequences proposed for the oxidation of formylphenylacetic acid ethyl ester, involving the formation of a substrate peroxide intermediate, also predicts that this reaction should not be inhibited by the dismutase enzyme.

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