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MONO- AND DIPHENOLASE ACTIVITY FROM FRUIT OF *MALUS PUMILA*

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Abstract—Apple fruit used for beverage production has a polyphenol oxidase which does not hydroxylate tyrosine under any conditions but it hydroxylates *p*-coumaric acid in the presence of NADH, and phloridzin in the absence of cofactors. The apparent K_ms for hydroxylation of phloridzin and *p*-coumaric acid are 1.5 and 4 mM, respectively. However, subsequent oxidation of 3-hydroxyphloridzin or caffeic acid has an apparent K_m of 200 nM. The oxidation products of 3-hydroxyphloridzin are complex and a stable dimeric quinone is finally formed. The apparent K_ms for oxidation of catechin, epicatechin, chlorogenic acid, L-Dopa and 4-methyl catechol are 4.7, 5.7, 6.0, 2.7 and 3.2 mM, respectively. The K_m for oxygen was 4.3% although there was marked substrate inhibition by oxygen above 30%. Polyphenol oxidase was stable at pH 3.5–4.5 with an optimum of 4.5.

INTRODUCTION

o-Diphenol:O_2 oxidoreductase (EC 1.14.18.1) catalyses both hydroxylation of *p*-monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones. The enzyme is ubiquitous in eukaryotes and hydroxylation of tyrosine in mammals has been rigorously investigated [1]. In particular, it has been shown that the enzyme has an absolute requirement for L-Dopa before hydroxylation takes place and there appear to be two catalytic sites, one for L-Dopa and one for tyrosine. There appears to be little, if any, competition for substrates between the two sites. In plants, Kahn and Pomerantz [2] have shown that the enzyme from avocado can hydroxylate tyrosine. Both this and the previous work used radiolabelled tyrosine, either ^{14}C or ^{3}H, to establish beyond reasonable doubt that hydroxylation was taking place. In the mammalian enzyme L-Dopa eliminated a lag phase [3], but in the case of avocado there was no reduction in the lag by tetrahydrofolic acid or DL-6-methyltetrahydropteridine [2]. However, Vaughan and Butt [4] found, with spinach beet phenol oxidase, that the lag in hydroxylation of *p*-coumaric acid to caffeic acid could be eliminated by *o*-diphenols together with an electron donor such as NADH, ascorbate or dimethyltetrahydropteridine.

The most widely investigated form of this enzyme is that from *Agaricus bisporus*. The original observations of copper in the protein [5] have been built upon until the structure of *Agaricus* enzyme is known to consist of two types of polypeptide chains, one with a MW of 43 000 and one with a MW of 13 400 [6]. The fungal enzyme will hydroxylate tyrosine without any cofactors but has been shown to show increased activity when extracted with detergent [7]. This latter increase in activity has frequently been reported with higher plant enzymes and the resulting preparations show association and dissociation phenomena [8].

Despite these findings, and the fact that plant diphenol oxidases are generally of broad substrate specificity, the enzyme extracted from apple fruit consistently appears to be unable to hydroxylate tyrosine [9]. However, other monophenols can be hydroxylated. Two pieces of evidence have indicated that *p*-coumaric acid undergoes very slow hydroxylation [9, 10] Raa and Overeem [11] have shown that the glycosylated monohydroxy phenolic phloridzin can be converted to the dihydroxy 3-hydroxyphloridzin, but in this case the glucose residue was first removed with a glucosidase.

In this paper we report the preparation of an enzyme from cider apple fruit (*Malus pumila* cv Dabinett) and the use of an alternative to the radiolabel method for establishing the apparent kinetic constants for both the hydroxylation and oxidation reactions. We have identified the hydroxylation products of phloridzin and coumaric acid by NMR spectroscopy and HPLC.

RESULTS

Hydroxylation reactions

No reaction occurred with tyrosine even in the presence of the cofactors NADH, L-ascorbate or DL-6-methyltetrahydropteridine. *o*-Diphenols such as L-Dopa or catechin were not effective in catalysing hydroxylation. However, tyrosine does not inhibit the oxidation of *o*-diphenols at all.

The use of HPLC enabled us to show that in the presence of NADH (5 mM) there was hydroxylation of *p*-coumaric acid to caffeic acid. The caffeic acid was identified by co-chromatography and was not further oxidized in the presence of NADH. Ascorbate and DL-6-methyltetrahydropteridine did not allow hydroxylation of *p*-coumaric acid and the presence of an *o*-diphenol was

not more effective in the presence of NADH than NADH alone. There was a slight lag period in the hydroxylation which was not eliminated by any of the cofactors. Concentrations of even 0.1 mM *p*-coumaric acid inhibited *o*-diphenol oxidation [12]. The evaluation of peak height by HPLC gave an apparent K_m for hydroxylation of *p*-coumaric acid of 4 mM. The Hill coefficient was 1.1 (correlation coefficient 0.998) indicating that there was no co-operativity in the enzyme action.

When phloridzin was reacted with the enzyme and analysed by HPLC a single peak was initially formed but was rapidly followed by formation of other peaks and appearance of a yellow colour. However, the presence of 5 mM ascorbic acid enabled the accumulation of the original peak to occur without conversion to other compounds. This was thought to be 3-hydroxyphloridzin and up to 50% conversion was achieved, but after this other products began to appear. By following the formation of this peak it was possible to obtain a K_m value for the hydroxylation of phloridzin of 1.5 mM and a Hill coefficient of 1.03 (correlation coefficient 0.979). Raa and Overeem [11] have shown that the product of phloretin (the aglycone) hydroxylation is 3-hydroxyphloretin. The single peak obtained on the HPLC was analysed by ^1H and ^{13}C NMR spectroscopy which showed that hydroxylation of phloridzin occurred on C-3 in ring B (Table 1).

Oxidation reactions

The oxidation of purified 3-hydroxyphloridzin was examined kinetically using either quantitation of peak size from the HPLC or by uptake of oxygen in the Clark electrode. Both methods gave a similar apparent K_m and this is shown in Table 2. Caffeic acid was also oxidized by the enzyme and the apparent constants are shown in Table 2. Catechin was oxidized to a coloured compound and both the appearance of compound (λ_{max} 390 nm) and uptake of oxygen were used to give the apparent K_m values (Table 2). The apparent K_m values and Hill coefficients indicate a very high affinity for compounds hydroxylated by the enzyme but much lower affinity for other diphenols. There appears to be a minimum of one binding site but previous evidence indicates that there is a separate binding catalytic site for hydroxylation in the mammalian enzyme [1].

Polymer formation after oxidation

The oxidation of 3-hydroxyphloridzin is accompanied by oxygen uptake and subsequently the quinone polymerizes to a coloured compound. Figure 1 shows the separation achieved by HPLC after oxidation and polymerization. Phloridzin (peak 4) and 3-hydroxyphloridzin (peak 3) are accompanied by a colourless compound (peak 1) and a coloured compound (peak 2). Neither peak 1 nor 4 (phloridzin) showed any *A* at 420 nm, whereas there was strong *A* at 280. In contrast peak 2 showed strong *A* at 420 nm and was an orange colour. Milligram quantities of this coloured material were prepared by chromatography on Sephadex LH-20 in water and crystallization from ethanol. The material exhibits λ_{max} 410 nm below pH 5.5 and λ_{max} 470 nm above pH 5.5 in accord with previous

Table 1. ^{13}C NMR spectral data of phloridzin and hydroxyphloridzin (50 MHz, DMSO-d_6)

C	Phloridzin	Hydroxyphloridzin	
		Observed	Calcd*
C=O	204.59	204.58	—
2′	165.27	165.21	—
4′	164.31	164.20	—
6′	160.71	160.69	—
4	155.13	—	—
4	—	144.72	142.4
3	—	142.98	141.8
1	131.42	132.19	132.8
2, 6	129.04 (2C)		
6	—	118.73	121.7
5	—	115.68†	116.3
2	114.88 (2C)	115.25†	116.3
1′	105.09	105.00	—
Anomeric (C-1 of glucosyl)	100.70	100.64	—
5′	96.75	96.66	—
3′	94.23	94.14	—
	77.16	77.12	—
	76.58	76.56	—
Glucosyl	73.11	73.00	—
	69.36	69.28	—
	60.45	60.42	—
CH_2CO	44.87	44.77	—
CH_2Ar	28.92	28.98	—

*Calculated using chemical shifts for phloridzin and substituent chemical shifts for hydroxyl.

†These assignments may be reversed.

Table 2. Affinity constants and Hill coefficients for substrates of *o*-diphenol oxidase of *Malus pumila*

Substrate	K_m(mM)	Hill coef.	Correlation coef.
Catechin	4.7	1.1	0.993
Epi-catechin	5.7	1.00	0.999
Chlorogenic acid	5.9	1.1	0.994
L-Dopa	2.7	1.0	0.999
Caffeic acid	0.2	1.1	0.991
4-Methyl catechol	3.2	1.0	0.995
3-Hydroxyphloridzin	0.2	1.0	0.995

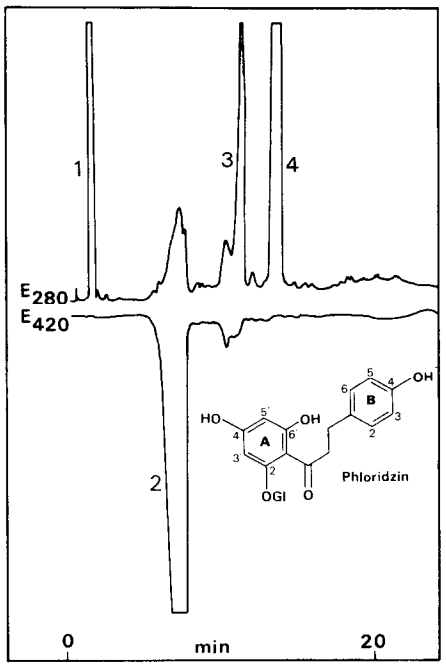

Fig. 1. HPLC separation of components after hydroxylation and oxidation of phloridzin. 1, colourless reaction product; 2, orange reaction product; 3, 3-hydroxyphloridzin; 4, phloridzin.

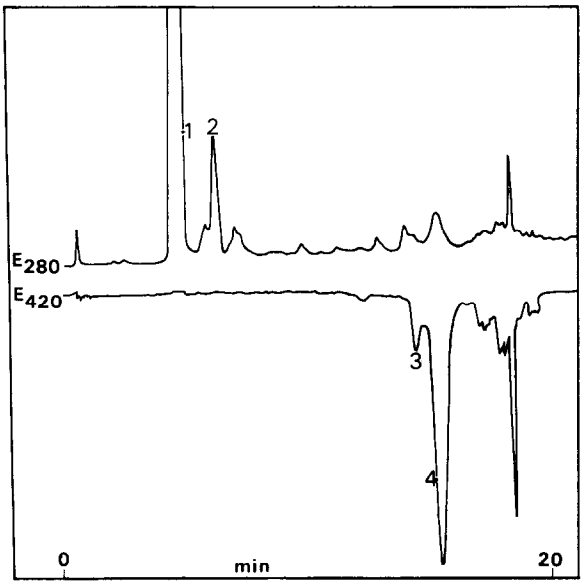

Fig. 2. HPLC separation of components after oxidation of catechin. 1, catechin; 2, presumed 8-hydroxy catechin [18]; 3, yellow product (presumed dehydrodicatechin A) [18]; 4, yellow stable oxidation product of catechin.

findings [3]. When subjected to ^{1}H and ^{13}C NMR spectroscopy using hydroxyphloridzin as a reference compound, evidence suggested the coloured compound was a mixture of two isomeric dimers, each linked through the B rings and incorporating additional oxygen. In both A rings the glucose moiety seemed to be still present. Fast atom bombardment mass spectroscopy indicated a MW of 954, supporting the proposed dimeric structure.

The oxidation of catechin was monitored by HPLC and showed a major peak of coloured material (peak 4) (Fig. 2). A λ_{max} of 390 nm indicated that this compound was the one monitored in the kinetic experiments. Since both oxygen uptake and appearance of the coloured compound gave the same affinity constant it would appear that there is a direct relationship between enzyme activity and production of coloured material.

Affinity for oxygen

Figure 3 indicates that oxygen can inhibit enzyme action at concentrations above 30%. A double reciprocal, plot of rate against substrate shows the normal curve for substrate inhibition. It is probably the first report of substrate inhibition by oxygen for a diphenol oxidase. The apparent K_m by extrapolation was 4.3% oxygen.

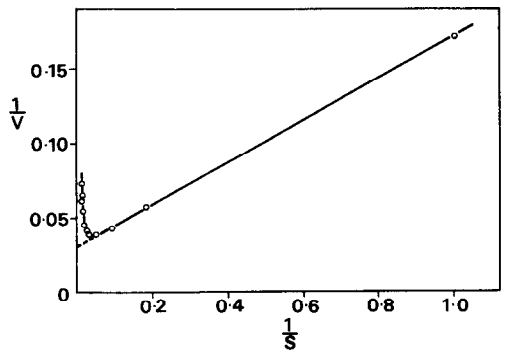

Fig. 3. Double reciprocal plot of velocity of enzyme activity with increasing concentration of oxygen. pH 4.5 citrate–phosphate buffer, 10 mM catechin, 30°.

pH optimum, pH and heat stability

The pH optimum for this enzyme was at pH 4.5 and fell rapidly to a minimum at pH 5.5. This gives the enzyme a lower pH optimum than previously reported for this species, but corresponds well with the very low pH of the ruptured tissue, which presumably reflects the vacuolar pH. When held at various pH values the enzyme was stable at pH 3.5 but less stable at pH values above 6.0. The enzyme was relatively stable to heat, losing 50% of activity after 10 min at 64°.

Polyacrylamide gel electrophoresis

All gels which were run and stained for the diphenol oxidase gave only one coloured band representing enzyme activity. The relative mobility to bromophenol blue was 0.6. This is unusual for the enzyme when many multiple forms are normally found but as this active preparation was obtained without detergent activation, it is entirely possible that the enzyme did not split into multiple forms.

DISCUSSION

The extraction procedure produced an enzyme capable of both hydroxylation of some monophenols and oxidation of several diphenols. When material from aged fruit was used to prepare acetone powders the enzyme was extractable using buffer only without the use of detergent unlike some other plant polyphenol oxidases [14]. However, the relative molecular mass of the protein was 26 000, this being close to the often quoted monomer size of plant *o*-diphenol oxidase (30 000) [8]. Demenyuk [15] observed three forms of diphenol oxidase in apple fruit, 24 000, 67 000 and 13 000 relative molecular mass but the higher form transformed into the lower forms during storage. We detected only the 26 000 form in aged fruit, it was active at this mass and only gave a single band of activity on polyacrylamide gels. The partially purified protein was stable at pH values of 3.5 but became more unstable at higher pH values. The enzyme showed a moderate stability at ambient temperatures but at low temperatures stability was good.

Use of the chromatographic procedures devised by Lea [16] enabled us to follow the hydroxylation reactions and estimate an apparent K_m value for them. In particular the K_m for phloridzin hydroxylation has not previously been obtained. It was noticeable that affinity for 3-hydroxyphloridzin and caffeic acid was much greater than for the other diphenols used. If the enzyme has two catalytic sites, as found in the mammalian enzyme, then it would seem that attachment of *p*-coumaric acid to the hydroxylating site partially blocks the oxidation site. This inhibition of oxidation by monophenols does not include tyrosine, which perhaps indicates that tyrosine does not bind to the enzyme.

Previous workers [17] have reported that maximum velocity is not achieved at oxygen concentrations ordinarily used in O–DPO measurements. We have not been able to confirm this observation for this enzyme. In fact oxygen concentrations greater than 30% inhibited enzyme activity. Whether this has anything to do with the fact that we were dealing only with an enzyme of relative molecular mass of 26 000, rather than an association of subunits, as reported by Demenyuk *et al.* [15], is a matter of speculation.

Sarapuu and Kheinara [13] have reported that oxidation of 3-hydroxyphloridzin results in a quinone, which complexes with phloridzin to form 6,5-(3-hydroxyphoridzyl-phloridzin), and this can exist as quinone–quinol isomers. The attachment of the B ring of oxidized phloridzin to the A ring of an oxidized phloridzin, as proposed by them, does not seem likely given the NMR and mass spectral data found by us, and at present it seems more probable that the units are linked through the B rings. Further structural studies are in progress.

EXPERIMENTAL

Preparation of enzyme extracts. Apple fruits were frozen in liquid N_2 and macerated in a blender with Me_2CO at −30°. The mixture was filtered through a Buchner funnel and rehomogenized ×4 with Me_2CO at −30° to remove all phenolics. The concn of phenolics was monitored by HPLC as described in a later section. The powders were lyophilized at −30° and protein was extracted by use of a pH 7.0 buffer containing 100 mM HEPES, 1 mM EDTA and 1% PVP(w/v). 20 ml buffer was added to 1 g Me_2CO powder, the mixture homogenized and filtered through muslin. The resulting supernatant was centrifuged at 20 000 *g* for 15 min and the clear soln remaining was made 100% satd with $(NH_4)_2SO_4$. After centrifugation at 20 000 *g* for 10 min, the pellet was dissolved in the pH 7.0 buffer and passed through a column (20 × 1.6 cm) of Sephadex G15. The eluate was recovered, lypohilized and redissolved in citrate–Pi buffer, pH 4.5. After passage through a Sephadex G100 column (27 × 1.3 cm, V_0 15.3 ml) the active fraction (V_e = 27.6 ml) was used for further tests. The Sephadex was calibrated using bovine serum albumin, chymotrysinogen A and cytochrome *c*. The relative molecular mass of diphenolase was 26 000 when compared to the standard proteins.

Enzyme assay. O_2 uptake was detected in a Clark electrode (Rank Bros. Bottisham, Cambridge) using the substrates and cofactors dissolved in 2 ml pH 4.5 citrate–Pi buffer at 30° and using 100 μl enzyme.

Increase in *A* at 390 nm was monitored using 3 ml substrate at pH 4.5 with 100 μl enzyme preparation at 30°.

Stability of enzyme samples. Enzyme preparations were kept at a series of pH values at 2° and tested for activity over a period of 11 days. At pH 3.5 80% of the activity was retained after 6 days and 25% after 11 days. The loss of activity at higher pH values was slightly less until pH 6.0 was reached when activity fell markedly over 4 days (30% retained).

Polyacrylamide gel electrophoresis. Slab gels (14 × 16 × 1.5 mm) were made using 7.5% acrylamide, pH 8.9, and a spacer gel of 2.5%, pH 6.9. Gels were subjected to electrophoresis at 3 mA/well (30 mA total) for 1 hr and 1 mA/well (10 mA total) for 3 hr at 2°. Completed gels were stained with 5 mM substrates (or satd solns if below 5 mM solubility) at 30°.

HPLC. Performed on a 100 × 5 mm column packed with Spherisorb hexyl 5μ (Phase-Separations) in a Spectra Physics SP 8000A instrument. Detection was at 280 and 320 nm (for phenolic acids) or 420 nm (for oxidation products) using a Pye LC3 detector for the visible detection. Solvents used were: (A) 0.1% $HClO_4$ in H_2O (pH 2.5); and (B) MeOH distilled from KOH. At a flow rate of 3 ml/min the solvent gradient was linear from 2% B to 30% B in 15 min, to 98% B in 5 min. The temp. was 45° and 20 μl samples were injected.

Preparation of 3-hydroxyphloridzin. Milligram quantities of pure 3-hydroxyphloridzin were produced by large-scale HPLC separation of the reaction mixture in MeOH–H_2O (2:3) on a Merck RP8 column (16 × 250 mm). The coloured oxidation product was purified by passage through an LH-20 Sephadex column (56 × 7 cm) using H_2O as solvent.

^{1}H *NMR* (200 *MHz*, *DMSO*-d_6). (a) Phloridzin: δ 2.81 (2H, *t*, J = 7.1 Hz, CH_2CO), 3.30–3.60 (*m*, glucosyl H, CH_2, Ar and H_2O), 3.70–3.76 (1H, *m*, glucosyl H), 4.66 (1H, *s*, glucosyl OH), 4.97 (1H, *d*, J = 6.8 Hz, anomeric proton, on C-1 of glucosyl), 5.10 (1H, *s*, glucosyl OH), 5.20 (1H, *s*, glucosyl OH), 5.35 (1H, *s*, glucosyl OH), 5.96 (1H, *d*, J = 1.9 Hz, ring A protons), 6.16 (1H, *d*, J = 1.9 Hz, ring A protons), 6.67 (2H, *d*, J = 8.3 Hz, H-3, H-5, ring B), 7.06 (2H, *d*, J = 8.8 Hz, H-2, H-6, ring B), 9.14 (1H, *s*, phenolic OH, probably B ring), 10.63 (1H, *s*, phenolic OH, probably C-4), 13.56 (1H, *s*, chelated OH, C-6). (b) 3-hydroxyphloridzin: δ 2.73 (2H, *t*, J = 6.8 Hz, CH_2CO), 3.20–3.60 (*m*, glucosyl H, CH_2, Ar and H_2O), 3.65–3.75 (1H, *m*, glucosyl H), 4.66 (1H, *s*, glucosyl OH), 4.94 (1H, *d*, J = 6.8 Hz, anomeric proton on C-1 of glucosyl), 5.09 (1H, *s*, glycosyl OH), 5.19 (1H, *s*, glucosyl OH), 5.32 (1H, *s*, glucosyl OH), 5.94 (1H, *d*, J = 1.95 Hz, ring A protons), 6.13 (1H, *d*, J = 1.95 Hz, ring A protons), 6.45–6.62 (3H, *m*, ring B protons), 8.68 (2H, *s*, phenolic OH probably B ring), 10.61 (1H, *s*, phenolic OH probably C-41), 13.54 (1H, *s*, chelated OH, C-6).

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