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GALLIC ACID OXIDATION BY TURNIP PEROXIDASE

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Abstract—Both ellagic and gallic acids non competitively inhibited guaiacol oxidation by turnip peroxidase. The K_i values were 3 and 26 μm for ellagic and gallic acid respectively. Enzymatic oxidation of gallic acid by the isolated major turnip peroxidase was characterized with respect to spectral behaviour, affinity constant and pH effect. The K_m for H_2O_2 and gallic acid are 2.5 and 8.0 mM for turnip peroxidase. The pH optimum for gallic acid oxidation is about 6.5 and the rate constant k_4 decreased with the increase of pH in presence of both guaiacol and gallic acid. When the gallic acid oxidation products were subjected to chromatographic analysis, it was found to be converted mainly to ellagic and an unknown quinone.

INTRODUCTION

Numerous physiological functions have been reported for peroxidases. In higher plants, its involvement in indole acetic acid oxidation (IAA) [1], lignification [2], oxidation of reduced pyridine nucleotides [3], and the production of ethylene from methionine [4, 5] have been described. Peroxidatic deamination of amino acids by crude extracts from terrestial isopods and fresh water crayfish [6, 7] and by myeloperoxidase from leukemic cells were also reported [8]. Potent antibacterial systems based on myeloperoxidases in phagocytes have been recently reviewed by Sbarra *et al.* [9]. All the above mentioned enzymatic reactions catalyzed by different peroxidases are either irreversible or can be catalyzed by alternative enzyme systems. The only peroxidase enzymatic activity which cannot be duplicated is the peroxidatic attack on oxidogenic donors e.g. *p*-cresol, *m*-cresol, guaiacol and resorcinol to form complex coloured products [10]. Characterization of isoperoxidases on the basis of their enzymatic activity on the the different phenolic and related compounds has been recently carried out [11–13]. However, in these studies gallic acid had never been examined as a possible hydrogen donor. Although gallic acid oxidation in the presence of peroxidase and H_2O_2 has been reported [14, 15], neither the enzymatic reaction nor the reaction products have been characterized. In this report, a study on the enzymatic oxidation of gallic acid (3,4,5-tri-hydroxybenzoic acid) by turnip peroxidase–hydrogen peroxide system and an attempt to identify the reaction products are presented.

RESULTS AND DISCUSSION

Some phenolic compounds (e.g. monophenols, resorcinol) are stimulatory; others are inhibitory (most polyphenols) for IAA or pyridine nucleotide oxidation despite the fact that all are substrates for peroxidase [16, 3]. Ferulic acid and scopoletin not only stimulate the guaiacol assay but also act as substrates for isoperoxidases from *Nicotiana tabacum* [11, 12]. On the other hand, uric acid, which is a well known substrate, competitively inhibits guaiacol oxidation by peroxidase A and B from *Cynara scolymus* ($K_j = 6.5 \times 10^{-5}$ and 1.46×10^{-4} M for A and B respectively) [17]. The inhibition of guaiacol oxidation by turnip peroxidase was examined in the presence of 0, 8, 16 or 25 μm gallic acid. Unlike uric acid, gallic acid acted as a non-competitive inhibitor for guaiacol oxidation (K_i 26 μm). The inhibitory effect of ellagic acid was tested at a concentration level ranging from 0–5 μm and was found to act as a non competitive inhibitor (K_i 3.3 μM).

Spectrophotometric studies on the oxidation of gallic acid

Addition of turnip peroxidase to assay reaction mixture containing gallic acid without guaiacol causes a rapid conversion of the acid to a new product which is yellowish in colour. The changes in the spectra during the turnip oxidation of gallic acid at pH 6.5 are examined. There are significant continuous increases in A with the increase of the incubation time, showing a maximum at 390 nm and around 305 nm. Unfortunately scanning the enzyme reaction mixture in the UV region was unsuccessful because of the high concentration of gallic acid. The change in A at 400 nm with time in the presence of different enzyme concentrations indicates that the rate of gallic acid oxidation is dependent on enzyme concentration and the formed chromogen is directly proportional to time up to 10 min. Also, it suggests that enzymatic oxidation of gallic acid can be assayed by following the change in A at 400 nm.

pH Optimum

Optimal pH for gallic acid oxidation by turnip peroxidase was determined by following the change in A at 400 nm. The rate of reaction is pH dependent and the optimal activity was achieved at pH 6.5–7, a known optimal range for peroxidase activity. Based on K_4 measurements [29] it was concluded that acidic horseradish peroxidase was consistently more active at pH 4.5 than at pH 7, whereas the slightly basic peroxidase showed the reverse behaviour in the presence of phenols [13]. With guaiacol and gallic acid as substrates, variation in pH affected the rate constant k_4 for turnip peroxidases (Table 1). Gene-

Table 1. Rate constants k_4 for the reaction between turnip peroxidase and hydrogen donors at different pH values

Buffer	pH	Guaiacol	Gallic acid
		k_4 ($\times 10^5$)	k_4 ($\times 10^5$)
Acetate	5.6	5.24	3.2
Phosphate	6.5	3.85	2.78
	7.5	2.57	1.35

rally turnip peroxidase showed higher k_4 values at pH 5.6 than at pH 7.5, however, the determined k_4 values indicate higher preferences towards guaiacol.

Peroxidase may act as oxygenase in presence of divalent metals and catalytic amounts of H_2O_2 [10]. However neither O_2 consumption nor CO_2 release in absence or presence of $MnCl_2$ was observed when the reaction was examined manometrically. Also, no change in A was observed when H_2O_2 was eliminated from the enzyme reaction mixture.

Apparent K_m for H_2O_2 *and gallic acid*

The initial rates of peroxidatic oxidation of gallic acid with H_2O_2 were determined at 25° in 0.05 M sodium phosphate buffer pH 6.5. Lineweaver–Burk plots of reciprocal initial velocity versus reciprocal initial concentrations of H_2O_2 and gallic acid resulted in linear relations from which the K_m for both substrates is derived. Under the specified conditions of temperature, pH and substrate concentration, K_m values of about 2.5×10^{-3} and 8.0×10^{-3} M were determined for H_2O_2 and gallic acid respectively.

Identification of gallic acid oxidation products by turnip peroxidase

The results of PC of gallic acid oxidation products in four solvent systems are shown in Table 2. Two spots out of the three major products detected on the chromatogram can be identified as gallic acid and ellagic acid. Ellagic acid fluoresces lemon yellow under UV after treatment with ammonia. Also, the UV spectrum of the eluted material showed typical maxima at 258 and 362 nm. The spot of the 3rd unknown product is yellow in colour and gives brown and greyish coloured spots with both sulphanilic acid and $FeCl_3$ spray reagents respectively. When the chromatogram was sprayed with the 2,4-dinitrophenyl hydrazine reagent, the yellow spot became deeper in colour with time and turned green after respraying with alcoholic NaOH [31]. After drying the colour of all spots changed to dark-brown. The spectrum of the eluted material showed sharp maxima at 390 and 305 nm. Quantitative PC indicated that under the standard assay reaction conditions, the amount of formed ellagic acid and the unknown product were 0.19 and 0.08 mg respectively (i.e. 2.4:1). Thus ellagic acid is a major product from the action of turnip peroxidase on gallic acid.

Tyramine and tyrosine in presence of HRP and H_2O_2 are oxidized to diphenyl compounds referred to as dityramine (2,2′-dihydroxy-5-5′-*bis*(B-ethylamine) diphenyl) and dityrosine respectively [18]. Also, di-*p*-cresol(2,2-dihydroxy-5,5′-dimethyl diphenyl) was isolated by Weterfeld and Lowe [19] from the reaction of *p*-cresol, peroxidase and H_2O_2. Thus, based on the reaction mechanism proposed for the formation of diphenyl compounds formation of ellagic acid from gallic acid by peroxidase H_2O_2 may be presented as follows:

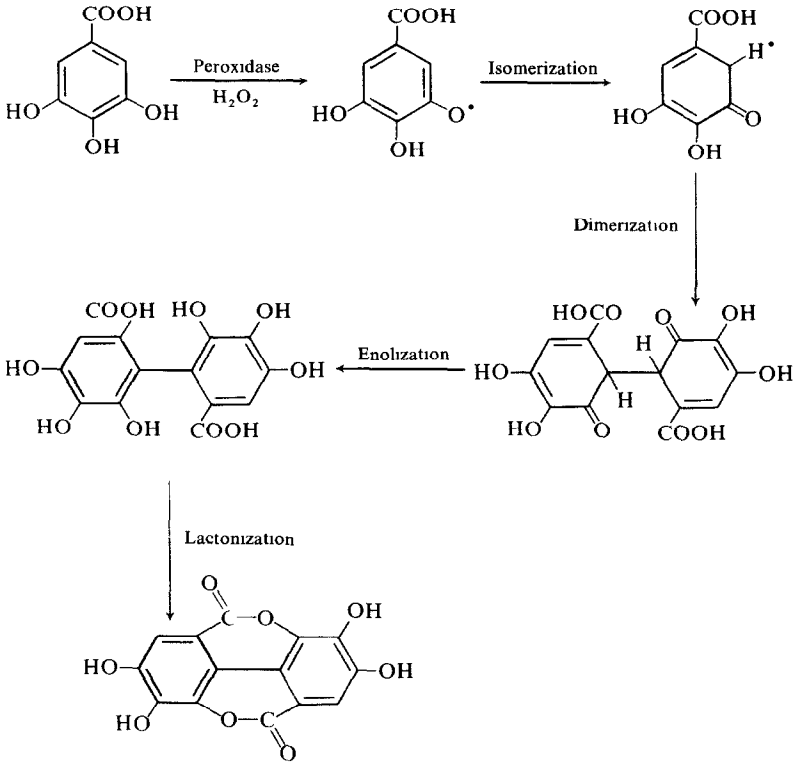

Table 2. Paper chromatographic R_f values of authentic gallic, ellagic and the product of gallic acid oxidation in different solvent systems

Solvent system	Gallic acid		Ellagic acid		unknown (yellow product)
	Standard	Isolated	Standard	Isolated	
I. *n*-BuOH–HOAc–H_2O (4:1:5)	0.70	0.69	0.34	0.33	0.76
II. 60% *iso*PrOH	0.80	0.79	0.27	0.24	0.66
III. 60% HOAc	0.81	0.80	0.36	0.36	0.65
IV. 15% HOAc	0.73	0.73	0.075	0.076	0.24

It had been suggested that peroxidases and the low MW *o*-dihydroxy phenols are important redox regulators in plant cells through their inhibition of peroxidase catalyzed oxidation of IAA or that leading to lignin formation [20–22]. Gallic acid may play such a role through its oxidation to ellagic acid which is a potent inhibitor for peroxidase catalyzed reactions.

Dihydroxy phenols are oxidized by peroxidase to *o*-quinones which undergo polymerization [23]. Based on the behaviour at alkaline pH, reactivity with 2,4-dinitrophenyl hydrazine, $FeCl_3$ and sulphanilic acid reagents, the yellow product may be identified as a quinone. In favour of such a proposal is the formation of 2,6-dimethoxyquinone by the action of peroxidase on α-methylsyringl alcohol and acetosyringone [24].

EXPERIMENTAL

Purification of turnip peroxidase. Peroxidase extracted from turnip roots was purified more than 230 fold by the methods previously reported [25, 26]. The purification steps included fractionation with $(NH_4)_2SO_4$ precipitation with Me_2CO, chromatography on TEAE cellulose, on cellulose phosphate and on Sephadex G 200. This preparation had sp. act. of 8600 units/mg protein and R.Z. 3.0; and showed a single band on disc electrophoresis [27].

Enzyme assays. Peroxidase activity was routinely assayed (unless otherwise stated) by measuring the change in *A* at 470 nm due to guaiacol oxidation in the presence of H_2O_2 and the enzyme at 30 sec intervals, according to the method described in [25, 26]. A unit of peroxidase activity was defined as that amount of enzyme which cause a change of 1 *A* unit per min at 25°. For gallic acid oxidation, the routine assay reaction mixture (unless stated otherwise) contained in a vol. of 3 ml: 8 μmol H_2O_2, 70 μmol NaPi buffer pH 6.5, 60 μmol gallic acid and enzyme at concns which gave a significant reading at 400 nm due to gallic acid oxidation after 2 min. The reaction was initiated by introducing the enzyme and mixing. All assays were carried out at 25°. Buffers used were prepared according to [28] k_4 was determined according to [29].

Sample preparation for chromatographic analysis. The routine reaction mixture was used and the reaction was initiated by introducing 0.35 units of enzyme and mixing; the increase in *A* was followed at 400 nm. Control reaction mixtures with no added enzyme or gallic acid were carried out simultaneously. The reaction after completion was acidified to pH 2 with HCl. The oxidation product was extracted with EtOAc (×3), the extracts combined, evaporated to dryness and the residue dissolved in a minimum vol. of EtOH.

Chromatography. Samples were spotted on Whatman No. 1, and gallic acid and ellagic acid as standards were also spotted on the same paper. Solvents used for developing the chromatograms had the following compositions: I. *n*-BuOH–HOAc–H_2O water (4:1:5). II. 60% *iso*PrOH. III. 60% HOAc. IV. 15% HOAc. The developing time for each chromatogram was 15–20 hr using ascending chromatography. Spots were located under UV and visualized by sulphanilic acid [30], $FeCl_3$ [31] and 2,4-DNPH [32] spray reagents. For quantitative determination the chromatograms were developed with 60% *iso*ProH and the ellagic acid spots were located on the chromatograms under UV after exposure to NH_3 vapour. Located spots were eluted in a known vol. of 0.05 M NaPi buffer pH 6.5. An authentic ellagic acid sample with known concn was chromatographed under the same conditions. *A* of the eluates was read at 362 nm and ellagic acid concns were calculated. The concn of the formed yellow product were calculated from the *A* at 400 nm from a previously established standard curve. When gallic acid was replaced by 1 μmol of ellagic acid, a new yellow spot with $R_f = 0.71$ was located under UV after exposure to NH_3 vapour. This spot was not detected in the controls, which is an indication that ellagic acid could act as a substrate.

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