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THE SUBSTRATE PROFILES OF THE ACIDIC AND SLIGHTLY BASIC HORSERADISH PEROXIDASES

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SUMMARY

1. The catalytic properties of two horseradish peroxidases, isoenzymes A2 and C2, with p*I* 3.9 and 8.8, have been compared.

2. The rate of formation of Compound I has been determined for the hydrogen, methyl, ethyl, *n*-propyl, and hydroxymethyl hydroperoxides and for *p*-nitroperoxybenzoic acid. The elongation of the alkyl group hampered the reaction with the acidic peroxidase and stimulated the reaction with the basic peroxidase.

The acidic peroxidase generally reacted more slowly. Its reaction with H_2O_2 showed a higher energy of activation than that of the slightly basic peroxidase.

3. The rate of reaction with hydrogen peroxide was slightly but definitely influenced by pH for both peroxidases.

4. The rates of reaction of twelve hydrogen donors (phenols, aromatic amines, acidic substances) with the secondary peroxidase–peroxide complex of the two peroxidases could be arranged in three groups according to the chemical nature of the donors.

The acidic peroxidase was consistently more active at pH 4.5 than at pH 7.0 whereas the slightly basic peroxidase showed the reverse behavior in the presence of phenols.

5. It is concluded that the two peroxidases are kinetically different with distinct substrate profiles and that they may fulfil different physiological functions.

INTRODUCTION

As revealed by electrophoresis and chromatography [1] plants possess a large number of peroxidases which occur in most, perhaps all, organs and differ in character from acidic, p$I < 4$, to strongly basic, p$I \geq 11$. Variations in origin, methods of isolation, and activity tests hamper a comparison, and the extraction of general properties to yield a classification according to functions is not yet possible.

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The reactions between a peroxidase (E), a peroxide, and an oxidizable substance (AH_2) are collected as

$$E + H_2O_2 \xrightarrow{k_1} \text{Compound I}$$

$$\text{Compound I} + AH_2 \xrightarrow{k_7} \text{Compound II} + AH\cdot$$

$$\text{Compound II} + AH_2 \xrightarrow{k_4} E + AH\cdot$$

k_7 is usually 40–100 times larger than k_4, both velocity constants being considerably less than k_1.

For a neutral horseradish peroxidase, Chance [2, 3] has determined k_1 with hydrogen, methyl, and ethyl hydrogen peroxide [2] and k_4 with ascorbate, hydroquinone, pyrogallol, guaiacol, and leucomalachite green [3]. The rate of reaction was markedly higher at low pH with ascorbate and at neutral pH with guaiacol. This horseradish peroxidase preparation had been purified electrophoretically at pH 10.6 and subsequently crystallized, the existence of subfractions being unknown at that time. An acidic, chromatographically separated horseradish peroxidase showed only one third the overall activity of a neutral horseradish peroxidase against guaiacol [4]. Four subfractions of a slightly basic commercial horseradish peroxidase were equally active against *o*-dianisidine and also against 2-methyl-1,4-naphtohydroquinone [5]. Four acidic peroxidases differed from four basic peroxidases in a Mn^{2+}-dependent oxidatic reaction with oxaloacetate and in a peroxidatic reaction with *o*-dianisidine [6]. The predominant peroxidase in Alaska pea seedlings has a somewhat higher k_1/k_4 ratio than the predominant root peroxidase and a 40% higher ratio between the rates of oxidation of *o*-dianisidine and guaiacol. Its activity as an indole acetic acid oxidase was, however, lower when related to the guaiacol peroxidase activity [7]. Two acidic, one neutral, and one basic peroxidase from Japanese radish gave nearly the same k_1 with H_2O_2 whereas their k_4 with guaiacol varied 15-fold, the acidic peroxidase being the least active [8]. For the subfractions of rice embryo peroxidase the same laboratory reported 200-fold variations in k_1 with H_2O_2 and 15-fold variations in k_4 with guaiacol, again the acidic peroxidase being least active in both reactions at pH 7 [9].

The peroxide and hydrogen donor profiles of the two quantitatively predominant plant peroxidases of the A and C types* have never been systematically elucidated. The present paper compares the reactions with six hydroperoxides and some traditional hydrogen donors and the effects of changes in acidity, temperature and solvent composition. Part of the results have appeared in a preliminary communication [10].

METHODS AND MATERIALS

k_1 was determined in a Durrum Gibson stopped-flow spectrophotometer.

k_4 was determined by the following four procedures, chosen according to the

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* In previous papers we denoted the two main horseradish peroxidase fractions by Ib and IIIb. The numbers originated as chromatographic fractions and slipped, regrettably, into print. To conform to usage, A and C are now reintroduced for I and III, A2 and C2 corresponding to Fractions Ib and IIIb, respectively.

actual situation and the rate of the reaction, so as to make this step rate-limiting. (a) For hydrogen donors with $k_4 < 10^5\ M^{-1}\cdot s^{-1}$, by steady-state measurements on Compound II [11]. (b) For donors with reasonably high reaction rates and stable products (e.g. guaiacol), k_4 was obtained from the linear steady-state increase in the light absorption of the final products [12]. (c) The originally present hydrogen donor decreases as a result of two reactions, with the velocity constants k_7 and k_4. Hence, for donors with high reaction rates and uncoloured products, k_4 equals half the pseudo-first-order rate constant for the decrease in the absorption of the donor, the presumption being made that $k_7 \gg k_4$ and that no disproportionation of the primarily formed radical occurs. It was checked that the actual absorbance values were not influenced by late products; semilogarithmic plots gave straight lines. (d) For the same reason, k_4 could be taken as half the rate constant for the pseudo-first-order approach to an approximately constant level in absorbance due to coloured products. These values (d) are minimum values but the error is estimated to be much less than a factor of 2, which is sufficient for the present purpose. The reproducibility of all four procedures was acceptable, the accuracy decreasing from (a) to (d). It was checked that the rates of the reactions were proportional to the enzyme concentration for (b), (c), and (d).

Horseradish peroxidase isoenzymes A2 (p*I* 3.9) and C2 (p*I* 8.8) were isolated as described [13].

H_2O_2 ("Perhydrol", Merck) was standardized against $KMnO_4$. Methyl- and *n*-propylhydroperoxide were synthesized [14, 15]. Ethylhydroperoxide was obtained from AB Ferrosan, Malmö, and *p*-nitroperoxybenzoic acid from Fluka AG. Hydroxymethylhydroperoxide was prepared [16] and freed from H_2O_2 by means of catalase [17]. Actual concentrations of the peroxides in water solutions were assayed via horseradish peroxidase peroxidation of dicarboxidine (AB Kabi, Stockholm) or guaiacol (at pH 7.0 and 4.5, respectively). These reactions were standardized against H_2O_2. The H_2O_2 content of the peroxides was $\leqslant 1.6\%$ as determined by means of Ti (IV) [16].

Chloroform extracts of the reaction mixtures were concentrated and subjected to thin-layer chromatography on silica gel "Nach Stahl, H 254" with chloroform–benzene (7:3, v/v) or ethylacetate–benzene (1:10, v/v). The chromatograms were inspected under ultraviolet light, sprayed with vanillin–H_2SO_4, and developed at 140 °C for 10 min. Acetylation prior to thin-layer chromatography did not improve the resolution or change the conclusions.

For other details, see Ohlsson and Paul [18].

RESULTS AND DISCUSSION

Nature of final product

Thin-layer chromatograms of the products from the reactions (0.5–5 h) between H_2O_2 and *N,N*-dimethyl-*p*-toluidine, guaiacol, or coniferol gave the same pattern with both peroxidases as catalysts. Leucomalachite green, on the other hand, gave different product patterns with horseradish peroxidase A2 and C2, independent of the concentrations of the enzymes. Triphenylmethane after treatment with H_2O_2 and either peroxidase gave only one spot on thin-layer chromatograms, corresponding to unchanged material. In spectrophotometric studies leucomalachite green reacted with H_2O_2 in a molar ration of 1:1 to form a coloured product with a A_{max} of approx. 600 nm. This substance was further converted to a species with a A_{max} of approx. 440 nm in a reaction that seemed to consume more than 1 molecule of H_2O_2 per original donor molecule. Both reactions were peroxidase catalyzed, horseradish peroxidase C2 being approximately 10 times more efficient than A2 in both. It was obvious that several reactions occurred consecutively and in parallel during the oxidation of leucomalachite green and that a complete analysis would be an extensive task. The present results are compatible with different reaction mechanisms for the two peroxidases, not accounted for by the reaction rates.

Peroxide substrates

Various hydroperoxides give identical compounds with horseradish peroxidase of type C [19–21], and the active site on this peroxidase is considered identical for all oxidizing agents [21]. Horseradish peroxidase C2 gives essentially the same k_1 values with H_2O_2, methylhydroperoxide and ethylhydroxperoxide (Table I) as those reported by Chance [2], which establishes a near similarity between the enzyme preparations

TABLE I

RATE CONSTANTS FOR THE FORMATION OF HORSERADISH PEROXIDASE–PEROXIDE COMPOUND I, AND THE EFFECT OF pH ON THE RATE CONSTANT WITH H_2O_2

0.4–0.6 μM peroxidase. 397 nm, 25 °C. 10 mM Tris–HCl, pH 9.0, 10 mM sodium phosphate, pH 7.0, or 10 mM sodium acetate, pH 4.5. Hydroxymethylhydroperoxide is rapidly hydrolysed at a higher pH. p-Nitroperoxybenzoic acid was found to give a pK_a of 7.3.

Hydroperoxide	μM	$k_1 \times 10^{-6}$ ($M^{-1}\cdot s^{-1}$)			pH
		Horseradish peroxidase:	A2	C2	
		Isoenzyme type:	pI 3.9	pI 8.8	
H_2O_2	6.4		1.4	12.0	4.5
H_2O_2	6.4		2.0	14.6	7.0
H_2O_2	6.4		1.1 (1.7)	13.0	9.0
Methylhydroperoxide	5.4		0.17	1.3	7.0
Ethylhydroperoxide	5.9		0.08	4.0	7.0
n-Propylhydroperoxide	8.9		0.037	4.8	7.0
Hydroxymethylhydroperoxide	10.9		0.011	0.45	4.5
p-nitroperoxybenzoic acid	4.9		0.47	37	4.5

The reactivity of horseradish peroxidase C2 with hydroperoxides is not negatively correlated to the size of the molecule (Table I,) the reactivity with the alkylhydroperoxides increasing with the length of the hydrophobic alkyl group. The high reactivity with nitroperoxybenzoic acid is in line with the tendency shown by the other hydroperoxides with markedly hydrophobic groups. The hydrophilic hydroxymethylhydroperoxide reacts only one tenth as fast as the roughly equally sized ethylhydroperoxide. We find some correlation between pK_a and k_1 for *p*-nitroperoxybenzoic acid, H_2O_2, and ethylhydroperoxide but the other alkyl peroxides and hydroxymethyl peroxide invalidate the correlation, in support of the conclusion of Schonbaum and Lo [20] that the outer oxygen does not engage in an unassisted nucleophilic displacement. The results support the notion [2] that the site of the reaction with the peroxide substrate is freely accessible in this peroxidase, and it seems plausible to interpret the effects as being caused by the partition of the peroxide between some hydrophobic site and water. This interpretation is also supported by the effects of changing the medium. Ethanol reduces k_1 with both dihydrogen and alkylhydrogen peroxides whereas high ionic strength increases k_1 with the alkylhydrogen peroxide (Fig. 1). From amino acid sequence analyses and analogies with other haemoproteins, Welinder [22] proposed the existence of a hydrophobic pocket in horseradish peroxidase of type C, able to accomodate both peroxide and hydrogen donor.

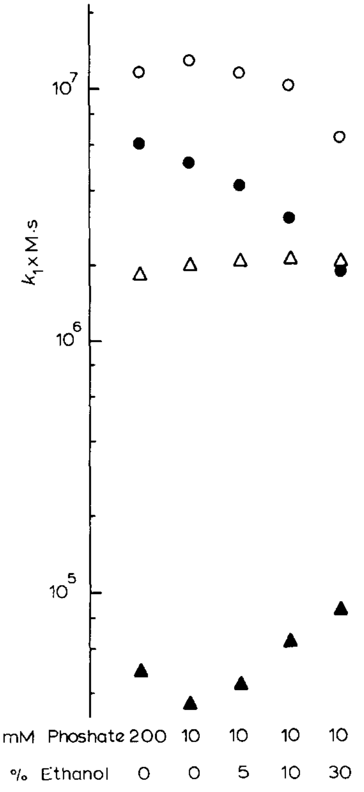

Fig. 1. Effects of high ethanol or phosphate concentrations on k_1. Horseradish peroxidase A2 (triangles) 0.51 μM, C2 (circles) 0.44 μM. H_2O_2 (open symbols) 4.3 μM, *n*-propylhydroperoxide (filled symbols) 4.6 μM. 397 nm, 25 °C, pH 7.0. The dimensions of the symbols are approximately equal to the uncertainty of the numerical values.

Horseradish peroxidase A2 generally yields lower k_1 values (Table I) than C2 and the activation energy for the formation of Compound I from H_2O_2 is twice as high for A2 as for C2, both values being low (Table II). The increasing length of the alkyl group decreases the k_1 of horseradish peroxide A2, opposite to the effect on C2, and the rate of reaction with hydroxymethylhydrogenperoxide, as compared to ethylhydrogenperoxide, is low also for A2. Finally, the effect of ethanol on k_1 for A2 is essentially opposite to that on C2 (Fig. 1).

TABLE II

THE EFFECT OF TEMPERATURE ON k_1 (6.4 μM H_2O_2)

10 mM sodium phosphate, pH 7.0. 397 nm.

Horseradish peroxidase 0.4–0.6 μM	$k_1 \times 10^{-6}$ ($M^{-1} \cdot s^{-1}$)			Activation energy (kJ·mole^{-1})
	At 10.0 °C	At 25.0 °C	At 40.0 °C	
A2, p*I* 3.9	1.5	2.0	2.9	17
C2, p*I* 8.8	11.9	14.6	17.2	9

Taken together, all this points to differences in the nature of the sites reacting with peroxides in the two peroxidases. The reactive site may be somewhat less accessible in horseradish peroxidase A2 than in C2, although both are probably quite exposed. As judged from the result with hydroxymethylhydrogenperoxide, increased hydrophilicity is not markedly decisive.

A characteristic feature of peroxidases and catalases is their high rate of reaction with hydrogenperoxides to form Compound I as compared to that of other haemproteins and haematin complexes possessing peroxidase activity. This high rate of reaction does not have a strict requirement for a uniform structure at the specific site, as previously evidenced by the structural differences between catalase and a peroxidase [23] and now between two plant peroxidases.

Hydrogen donors

Three types of donors were studied: phenols, aromatic amines and a somewhat heterogeneous group of acidic substances. Their reactions with the two peroxidases could be arranged into three groups which corresponded to the chemical classification (Table III). The slightly basic C2 was more active against the aromatic amines whereas A2 in general reacted faster with the acidic donors. With phenolic substrates variations in pH affected k_4 in opposite ways for the two peroxidases. Thus horseradish peroxidase A2 gave a higher k_4 at pH 4.5 than at pH 7.0 whereas C2 showed the reverse effect. The two ortho-methoxylated phenols reacted rapidly with both peroxidases, horseradish peroxidase A2 being more efficient at pH 4.5 and C2 at pH 7.0. In the range pH 4.5–7 the phenols will not protolyse [24, 25].

Thus horseradish peroxidase A2 and C2 show kinetically different preferences also towards the hydrogen-donating substrates, albeit with nearly identical reaction velocities with the growth regulator indole acetic acid. The grouping of the donors according to their reactivities is consonant with the observation of Fridovich [26] that the peroxidation of dianisidine and *p*-phenylenediamine, but not of guaiacol, uric acid, or hydroquinone, was stimulated by the addition of some nitrogeneous substances.

The velocity constants of the pairs guaiacol–coniferol and *N*,*N*-dimethyl-*p*-toluidine–leucomalachite green as well as of the three dihydric phenols confirm [2, 27] that the size of the hydrogen donor has little or no influence on the rate of the reaction with either peroxidase. As a matter of fact, a higher homologue of *p*-alkyl-aniline has been found to react faster than a lower homologue in batch experiments (Saunders, B.C., personal communication). Recent spectrophotometric [25] and kinetic [28]

TABLE III

RATE CONSTANTS (k_4) FOR THE REACTION BETWEEN PEROXIDASES A2 OR C2 AND HYDROGEN DONORS

a–d refer to descriptions in Materials and Methods. Buffers, 10 mM sodium acetate, pH 4.5, 10 mM sodium phosphate, pH 7.0.

Substrate hydrogen donor	$k_4 \times 10^{-4}$ ($M^{-1} \cdot s^{-1}$)				Method	Wavelength (nm)	Donor concn (μM)
	at pH 4.5		at pH 7.0				
	A2	C2	A2	C2			
1,2-Dihydroxybenzene (catechol)	15	28	6.3	29	(d)	387	145
1,3-Dihydroxybenzene (resorcinol)	4.1	17	2.6	22	(d)	282	145
1,4-Dihydroxybenzene (hydroquinone)	48	210	23	590	(c)	288	184
Guaiacol	54	23	18	52	(b)	470	310
Coniferol	1600	280	100	890	(c)	262	14
Leucomalachite green	≈1	≈10	–	–	(d)	600	6
N,*N*-Dimethyl-*p*-toluidine	1.70	12.0	–	–	(d)	340	46
Mesidine	1.9	8.5	1.2	2.8	(d)	285	61
Benzidine	1800	4900	140	4300	(c)	278	30
Ascorbic acid	19	1.4	2.1	0.05	(a)	427	200
Uric acid	3.9	1.2	1.2	1.9	(c)	284	54

analyses point at the existence of enzyme–donor complexes, which, however, do not yet seem to have found their definite roles in the enzymic reactions.

Schonbaum [28] demonstrated the binding of hydrophobic hydroxamic acids to horseradish peroxidase type C by hydrophobic (and hydrogen) bonds as well as the competition between such hydroxamic acids and the hydrogen donor for a site on the peroxidase. An assumption that this site is involved in the peroxidatic mechanism is in line with the results in Fig. 2. For horseradish peroxidase C2, ethanol decreases k_4 for the hydrophobic donor mesidine and increases k_4 for the hydrophilic ascorbate. With horseradish peroxidase A2, k_4 for both donors decreases, and in an identical way, when ethanol is added to the medium.

Haematin surroundings

The two peroxidases differ widely in amino acid composition [13] but have in common the prosthetic group, the molecular weight, and the absorption spectrum which is essentially of the high-spin type at room temperature. Both give a Soret band maximum at 403 nm but the absorbtivity of horseradish peroxidase A2 is 15% higher [13]. There is a strong, positive Cotton effect at approx. 400 nm in both peroxidases, but a negative Cotton effect in the near ultraviolet is more pronounced for horseradish peroxidase type A [29]. Further evidence for different haematin surroundings in horseradish peroxidase A2 and C2 are given by the redox potential, which is about 50 mV higher for horseradish peroxidase A2 (Ohlsson, P.-I. and Paul, K.-G., unpublished) and by the difference in pK_a for the shift from the neutral to the alkaline

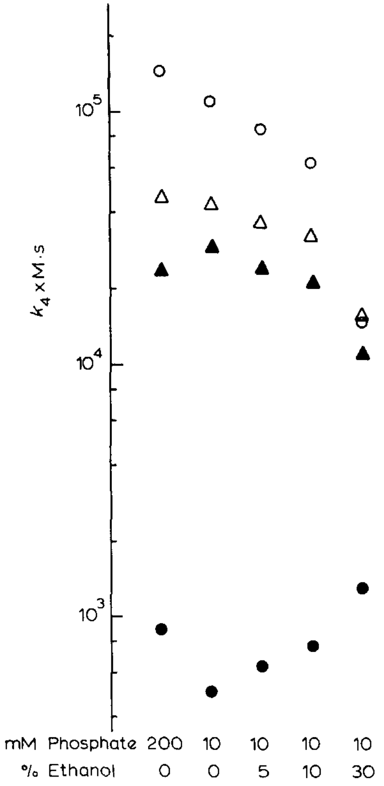

Fig. 2. Effects of high ethanol or phosphate concentrations on k_4. Horseradish peroxidase A2 (triangles) 0.11–0.28 μM, C2 (circles) 0.064–0.64 μM. H_2O_2 9–27 μM. Hydrogen donors mesidine (open symbols) 189 μM, ascorbate (filled symbols) 210 μM. 427 nm, 25 °C, pH 7.0. Procedure (a) for ascorbate, (d) for mesidine (cf. Methods). The dimensions of the symbols are approximately equal to the uncertainty of the numerical values.

form of the Fe^{3+} peroxidases. It occurred at pH 9.20 ± 0.02 (slope $n = 1.02 \pm 0.05$) for peroxidase A2 and at pH 11.1 ± 0.16 ($n = 0.98 \pm 0.05$) for C2, as measured at four wavelengths within the range 500–600 nm with plots of log $(A_\infty - A_{obs})/(A_{obs} - A_0)$ against pH. A pK_a value around 9 has been reported for other acidic peroxidases [8, 30]. In diacetyldeuterohaematin horseradish peroxidase C2 the replacement of the vinyl groups by diacetyl groups caused the lowering of the pK_a from 11.1 to 9,0, a change compatible with an altered electron distribution and a facilitated protolysis of water at Position 6 [18].

The alkaline form (Fe·OH) of the slightly basic subfraction of horseradish peroxidase is catalytically inactive [2]. An effect of pH upon the k_1 of the (Fe·H_2O) form was reported only recently and is seen also with other alkenyl-substituted haematin peroxidases [18]. There is a slight but definite effect on k_1 of both horseradish peroxidase C2 and A2 when the pH is raised from 4.5 to 7.0 (Table I). At pH 9.0 k_1 for C2 (pK_a = 11.1) is only 89 % of k_1 at pH 7. For A2 (pK_a = 9.2) the apparent k_1 value is 1.06, after correction for its inactive (Fe·OH) form 1.73, which is 87 % of k_1 at pH 7.0. Thus the two peroxidases respond quantitatively equally to the change from pH 7 to 9. In the experiments collected in Table I the reaction between horseradish peroxidase A2 and H_2O_2 at pH 9.0 followed a perfect first-order course until finished. This means that the conversion from the alkaline to the neutral form of horseradish peroxidase A2 must proceed considerably faster than does the formation of Compound I from the neutral form, contrary to what has been reported for a neutral horseradish peroxidase [31].

The binding site for the hydrogen donor is probably hydrophobic in horseradish peroxidase of the C type [21, 28]. Differences in binding site between horseradish peroxidase A2 and C2 are likely from the substrate profiles, and differences in hematin surroundings are obvious from the effects on pK_a and, presumably, apparent rates of protolysis.

A correlation of the hydrogen donor profile to the biological function requires knowledge of the cellular location of the two peroxidases.

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