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The inactivation of horseradish peroxidase by *m*-chloroperoxybenzoic acid, a xenobiotic hydroperoxide

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Abstract

m-Chloroperoxybenzoic acid (*m*-CPBA) acts as an oxidant substrate of peroxidase (EC 1.11.1.7) and, at the same time, is a powerful suicide substrate of the enzyme. A value for the partition ratio (r) between the catalytic and the inactivating routes is calculated in the absence of the typical reductant substrates of peroxidase: One mole of enzyme gives around two turnovers ($r = 1.8 \pm 0.1$). The kinetic analysis allows us to calculate a value for the inactivation constant $k_i = (4.80 \pm 0.40) \cdot 10^{-3}\ s^{-1}$, being very similar to that obtained for H_2O_2. These results suggest that, contrary to H_2O_2, in the case of *m*-CPBA a catalase-like reaction is not active and so the enzyme is not protected. Also, the calculated value for K_2 (6.54 μM) indicates a high affinity of Compound I for *m*-CPBA.

Keywords: Peroxidase; Kinetics; Inactivation; Hydroperoxide

1. Introduction

The enzyme peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) catalyzes the global reaction: $2AH + H_2O_2 \rightarrow 2A^{\cdot} + 2H_2O$, where an oxidant ($H_2O_2$) and a reductant (AH) react together. The opposing characters of the substrates determines that higher plant peroxidases can be classified into two groups, according to their functions. Thus, following Welinder's nomenclature [1]:

Class I: (typical representative: **ascorbate peroxidase**) are responsible for H_2O_2 elimination. In this case the limiting factor is the reductant substrate.

Class III: (typical representative: **horseradish peroxidase**) are secretory enzymes needed for polymerization reactions through free radical formation. In this case the limiting factor must be the peroxide.

The first step in the mechanism of peroxidation is the reaction of the enzyme with the oxidizing agent (either H_2O_2 or some other hydroperoxide), yielding the catalytic intermediate called Compound I. This is not an enzyme–peroxide complex but a reactive intermediate form with a higher oxidation state in comparison to the native enzyme [2–4]. Thus, Compound I can oxidize a large variety of reductant substrates [5], including additional molecules of hydroperoxide [6,7]. The

Abbreviations: ABTS: 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulphonic acid); *m*-CPBA: *m*-Chloroperoxybenzoic acid; *m*-CBA: *m*-Chlorobenzoic acid; HRP: Horseradish peroxidase

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many factors which influence the kinetics of Compound I formation can be characterized by varying the pH and/or the properties of the substituent in the hydroperoxides. These influences vary in importance depending on the enzyme source and, in enzymes from the same source, on the different isoenzymes [8–10].

Both classes of peroxidase differ in their sensitivity to hydroperoxides. Those of Class I need a reductant environment to be preserved [11], while those belonging to Class III can resist higher H_2O_2 concentrations, although the oxidant/reductant ratio will determine the number of catalytic turnovers of the enzyme [12,13]. It has been proposed that this inactivation process takes place at the level of Compound I [14,15] and the type of substituent in the hydroperoxide is again important for the efficiency of the inactivation.

With respect to Class III enzymes, we have already described the inactivation of horseradish peroxidase (HRP) by H_2O_2 [7] and its protection by the reducing agent 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), the oxidation product of which does not inactivate the enzyme [12,16].

In the present work, instead of H_2O_2, *m*-chloroperoxybenzoic acid (*m*-CPBA), is chosen as oxidant and inactivator substrate, for the following reasons:

1. it belongs to the interesting group of peroxybenzoic acids, which are efficient oxidants of peroxidase, releasing the parent carboxylic acid [17,18]:

   Enzyme + R–CO–OOH

   → CompoundI + R–CO–OH;

2. it possesses a bulky and characteristic substituent group, which permits a comparative study of its properties as inactivator [8,18].
3. *m*-CPBA cannot complete a catalase cycle when it reacts as a substrate of HRP [19–21], and so a different inactivation mechanism with respect to H_2O_2, can be proposed.

A kinetic study on the reaction of HRP with *m*-CPBA, in the absence of other reductant substrates, is made. The type of inactivation produced by *m*-CPBA is discussed.

2. Materials and methods

Purified horseradish peroxidase (HRP) was obtained from Sigma, type IX, RZ (A_{403nm}/A_{275nm}) = 3.1. The enzymatic sample was characterized by isoelectrofocusing as described previously [22]. The enzyme concentrations were estimated by measuring the absorbance of solutions at 403 nm using $\epsilon_{403nm} = 100\ mM^{-1}\ cm^{-1}$ [23].

m-Chloroperoxybenzoic acid (*m*-CPBA) in the solid state was supplied by Aldrich Chemical Co., containing *m*-chlorobenzoic acid (*m*-CBA) as an impurity. Two samples of *m*-CPBA were used and, according with the supplier, their purity was: (a) lot no. 3800511, purity between 80–90%; (b) lot no. 2361078, purity between 50–60%.

In our case, the actual purity was estimated by ^{1}H-NMR (300 MHz) using Varian equipment, model Unity. The samples (50 mg) were dissolved in $CDCl_3$ and TMS was used as a signal reference. The identification, in the sample spectra, of peak signals corresponding to *m*-CBA and *m*-CPBA was made using *m*-CBA (99% purity) as standard and was in accordance with the Aldrich Library of NMR spectra [24]. Thus, using this protocol, the actual amount of *m*-CPBA in the purchased samples was: (a) 75% and (b) 58%.

However, for the inactivation assays, the *m*-CPBA samples were purified by recrystallization in light petroleum ether (b.p. 40–60%)/diethyl ether (3:1, v/v) according to Davies et al. [18]. After this purification the product obtained presents a purity greater than 96% (NMR analysis and a single melting point at 92–94°C, corresponding to *m*-CPBA).

Solutions of *m*-CPBA were made up immediately before use. The concentration was estimated using a calculated $\epsilon_{232nm} = 8940\ M^{-1}\ cm^{-1}$, and the value was corrected by the purity factor obtained from NMR data.

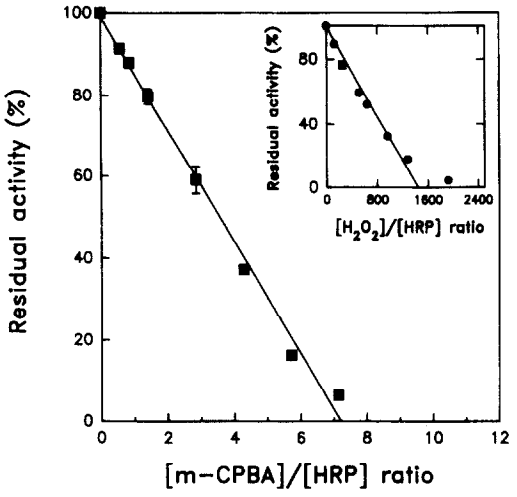


Fig. 1. Fractional enzymatic activity versus the [*m*-CPBA]/[HRP] ratio. The enzyme (0.26 μM) and the different concentrations of *m*-CPBA were incubated in buffered media (Na–phosphate buffer 50 mM, pH 6.5), and the residual enzymatic activity was assayed at the end-time of the reaction. The abscissa intercept value and Eq. 1, allow the calculation of *r* (number of turnover given by one mole of enzyme before its inactivation). Inset shows a similar approach but using H_2O_2 instead of *m*-CPBA.

Samples of *m*-CPBA without further purification were also used. Solutions prepared directly from lots (a) and (b) only were corrected by the NMR-purity factor, and assayed to measure the rate of HRP inactivation. Results similar to those obtained with purified *m*-CPBA were also obtained in every case with less purified *m*-CPBA samples. Moreover, the addition of pure *m*-CBA to a medium containing HRP did not produce any change in the enzyme spectrum and, also, the addition of *m*-CBA did not affect the ABTS oxidation catalyzed by HRP. Therefore, *m*-CBA did not seem to be an alternative substrate of HRP and so it had no effect on the reaction studied.

2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), in the form of crystallized diammonium salt, was supplied by Boehringer–Mannheim. The concentration of ABTS was estimated by measuring absorbance using $\epsilon_{340nm} = 36$ $mM^{-1}\,cm^{-1}$ [25]. Hydrogen peroxide (30%, v/v) was from Merck. The concentration of H_2O_2 was estimated by measuring absorbance using $\epsilon_{240nm} = 43.6\ M^{-1}\,cm^{-1}$ [26]. Buffer substances (analytical reagent grade) were also obtained from Merck. All solutions were prepare using deionized water obtained from a Milli-Q system (Millipore).

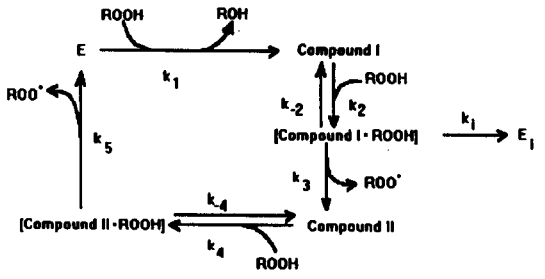
Spectrophotometric measurements were recorded with an UV–VIS Perkin-Elmer Lambda-2 spectrophotometer interfaced on-line with an Olivetti PCS/386SX computer. The temperature was controlled at 25°C using a Haake DIG circulating bath with a heater/cooler with a precision of ±0.1°C.

2.1. Rate of inactivation of peroxidase

The inactivation of HRP was carried out at 25°C in 1 ml incubations of 50 mM Na–phosphate buffer (pH 6.5) containing a fixed amount of the enzyme. The reactions were started by the addition of *m*-CPBA (over a range of concentrations). At specified time intervals, 10 μl aliquots of the incubation mixtures were transferred to cuvettes containing 2 ml of an assay mixture composed of 0.5 mM ABTS and 0.2 mM H_2O_2 in 50 mM Gly–HCl buffer (pH 4.5). The peroxidase activity was measured by the increase in absorbance at 414 nm, which is characteristic for the ABTS oxidation product [25]. A minimum of four incubation assays for each *m*-CPBA concentration were made. The residual enzymatic activity (A_R) was taken as the enzymatic activity (remaining) (A_t) with respect to the initial activity (A_0).

3. Results and discussion

In the absence of reductant substrate, the incubation of HRP with *m*-CPBA leads to a loss in enzymatic activity that depends on the incubation time and the *m*-CPBA concentration used [16]. To study this enzyme peculiarity, we can use an experimental approach developed to study the kinetics of suicide substrates [27,28]: this approach consists of estimating enzyme inactivation by measuring the residual enzymatic activity at the end-time of the reaction. Thus, a careful titration of the peroxidase with *m*-CPBA established that 6–8 equiv. of the hydroperoxide are required to maximally decrease the percentage of



Scheme 1.

residual activity (Fig. 1). When this result is compared with that obtained with H_2O_2 (Fig. 1, inset) *m*-CPBA appears to be a very effective inactivator of HRP.

The plot of the residual activity of the enzyme versus the substrate/enzyme ratio corresponds to the following equation (see Appendix 1):

$$A_R = \frac{A_t}{A_0} = 1 - \frac{1}{3r+2}\frac{[m-\text{CPBA}]}{[\text{HRP}]} \qquad (1)$$

where [HRP] is the initial concentration of the enzyme in the reaction medium; A_R is the residual enzymatic activity taken as the enzymatic activity (remaining) at the end of the reaction (A_t) with respect to the initial activity (A_0); [*m*-CPBA] represents the initial substrate concentration used for incubation; and *r* is a parameter defined as the number of turnovers given by one mole of enzyme before its inactivation [27].

Then, as Fig. 1 shows, an abscissa intercept appears, allowing one to calculate a value for *r*, according with Eq. 1. This value, obtained for *m*-CPBA, is $r = 1.8 \pm 0.1$. In the case of H_2O_2, a similar calculation (with data from Fig. 1 inset) would give a higher value.

It is interesting to note that with *m*-CPBA the value of *r* drops drastically, being reminiscent of that calculated when HRP, reacting with H_2O_2, follows the Compound III pathway (see Scheme 1 and data in [7]). In the case of *m*-CPBA, it seems that a catalase-like reaction is not active and so the enzyme is not protected because it cannot follow this alternative pathway.

The changes in the spectrum of native HRP observed during its reaction with *m*-CPBA, suggest the participation of intermediate active forms of the enzyme (Fig. 2). Experiments have been developed to observe the changes in the spectrum of HRP, when different *m*-CPBA/HRP ratios were assayed. Fig. 2 is representative of these changes when a 3:1 ratio is used. The initial spectrum of HRP changes very quickly. A decrease in absorbance in the Soret region (403 nm) is typical of the transition through Compound I. Over time characteristic spectra in the visible region with maxima at 523, 557 and 670 nm can be distinguished. The first two resemble the typical Compound II maxima, and the third corresponds to the verdohemoprotein P-670 which is a known inactive form of the enzyme [7,14]. The increase in absorbance with time for both wavelengths can be followed in the inset of Fig. 2, giving information about the rate of those reactions. Nakajima and Yamazaki [14] using *m*-nitroperoxybenzoic acid, instead of *m*-CPBA, obtained similar results and

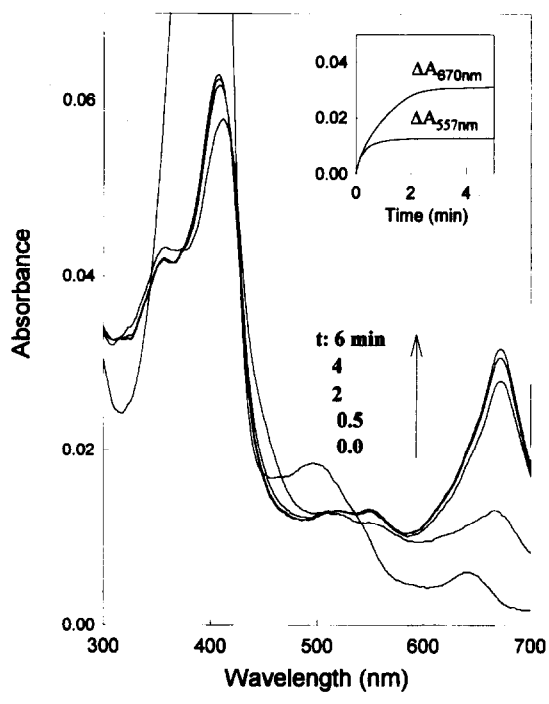


Fig. 2. Differences seen in the spectrum of HRP after incubation with *m*-CPBA. At zero time the spectrum of native peroxidase (1.5 μM) in 50 mM Na–phosphate buffer (pH 6.5) appears with $\lambda_{max} = 403$ nm in the Soret region and $\lambda_{max} = 500$ and 640 nm in the visible region. The successive traces represent changes at 0.5, 2, 4 and 6 min, respectively, after the addition of the equivalent amount of *m*-CPBA necessary to reach a [*m*-CPBA]/[HRP] ratio of 3:1. Inset shows changes in absorbance with time, at two wavelengths: ΔA_{557nm} (Compound II) and ΔA_{670nm} (P-670).

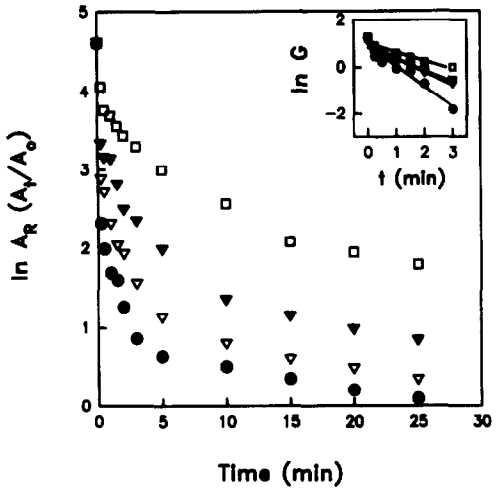


Fig. 3. Time-dependent inactivation of HRP by *m*-CPBA. The enzyme (0.1 μM) was incubated in buffered media with different concentrations of *m*-CPBA: Aliquots of each medium were withdrawn at the specified times, and the residual peroxidase activity was measured by the ABTS oxidation assay (see Materials and methods). The data are plotted in a semilogarithmic form. Inset shows the estimation for λ_1 and γ_1, according to the equation:

$$\ln\left(\frac{A_t}{A_0} - \gamma_2 e^{-\lambda_2 t}\right) = \ln\gamma_1 - \lambda_1 t; \text{ being } G = \left(\frac{A_t}{A_0}\right) - \gamma_2 e^{-\lambda_2 t}$$

The *m*-CPBA initial concentrations are: (□) 3.7; (▼) 7.5; (▽) 18.7 and (●) 37.5 μM. Each point represents the mean value of three different experiments.

gave additional information about the spectral changes using rapid-scanning and stopped-flow techniques. A careful addition of *m*-CPBA equivalents is necessary, otherwise the observation of intermediate enzymatic forms is not possible. In this sense, the difficulties of Adediran and Lambeir [29] to obtain Compound III of HRP from the reaction of Compound II with *m*-CPBA, can be justified.

In addition, the enzyme inactivation can also be studied by estimating the loss in peroxidase activity after different incubation times in the presence of *m*-CPBA. Thus, Fig. 3 shows the residual enzymatic activity (in a logarithmic trace) remaining after different incubation times of peroxidase with excess *m*-CPBA. The different traces, in Fig. 3, show also the dependence of the inactivation rate on the initial *m*-CPBA concentration present in each medium. As can be seen for each trace, the system evolves until there is a total loss of activity with an apparently bi-exponential behaviour.

Consequently and in order to interpret all the above experimental data, a minimum sequence for the reaction of peroxidase with *m*-CPBA can be postulated in Scheme 1. It shows, firstly, the reaction of native enzyme with *m*-CPBA (ROOH) yielding Compound I [8]. Then, a second molecule of *m*-CPBA can act as an electron donor giving rise to a (Compound I·ROOH) complex. From it, the partition is established:

1. one reaction produces Compound II and the *m*-CPBA free radical (ROO˙),
2. the other reaction leads to enzyme inactivation (E_i). Those previous reactions take place quickly, corresponding to the first phase in Fig. 3. The enzymatic forms prevailing are Compound II and P-670, as can be seen in Fig. 2.

The slow phase in Fig. 3, would correspond to the last steps in Scheme 1. The formation of a new complex (Compound II·ROOH) following the reaction of Compound II with a third molecule of hydroperoxide. Such a complex becomes Compound III when Compound II reacts with H_2O_2 [3,6]; however when *m*-CPBA is the reactant, instead of H_2O_2, the resulting compound has no currently defined name [14,29]. Finally, to close the typical peroxidative cycle, the (Compound II·ROOH) complex would decompose spontaneously to native peroxidase yielding a second molecule of *m*-CPBA radical (ROO˙).

Taking the above into consideration, a kinetic approach is possible. This system does not reach the steady state, but it evolves with time in the transient phase until total loss in enzyme activity occurs.

The kinetic equations characterizing the enzyme behaviour with time are developed according to the procedure described by Varón et al. (1990) [30] and following our previously published protocol for the suicide inactivation of HRP with H_2O_2 [7] (see Appendix B). The evolution of the system towards a total loss in enzyme activity can best be described by five-exponential term function. However, only two of these exponential terms appear significant with time, according to the results in Fig. 3. Consequently,

from the general solution it is possible to pass to a particular solution, in which the loss in enzyme activity could be described by the bi-exponential equation:

$$\frac{A_t}{A_0} = \gamma_1 e^{-\lambda_1 t} + \gamma_2 e^{-\lambda_2 t} \quad (2)$$

where: A_t and A_0 are the measures of peroxidase activity estimated after the incubation time t and zero, respectively; λ_1 and λ_2 are the apparent inactivation constants of the system; γ_1 and γ_2 are the amplitudes of exponential terms.

The apparent inactivation constants (λ_1 and λ_2) are the roots of the equation:

$$\lambda^2 - M_1\lambda + M_2 = 0 \quad (3)$$

where:

$$M_1 = \frac{(k_3+k_i+k_5)[\mathrm{ROOH}]_0^2 + \{(k_3+k_i)K_4 + k_5K_2\}[\mathrm{ROOH}]_0}{[\mathrm{ROOH}]_0^2 + (K_2+K_4)[\mathrm{ROOH}]_0 + K_2K_4} \quad (4)$$

$$M_2 = \frac{k_ik_5[\mathrm{ROOH}]_0^2}{[\mathrm{ROOH}]_0^2 + (K_2+K_4)[\mathrm{ROOH}]_0 + K_2K_4} \quad (5)$$

Between λ_1 and λ_2 the following relationships are established:

$$\lambda_1 + \lambda_2 = M_1 \quad (6)$$

$$\lambda_1\lambda_2 = M_2 \quad (7)$$

If we now assume that:

$$k_5 \ll k_3 + k_i \quad (8)$$

then, we have, taking Eqs. 4 and 5 into account,

$$M_1^2 \gg M_2$$

and, therefore, the relationship

$$M_1^2 \gg 4M_2 \quad (9)$$

must also be true. Inserting Eq. 9 into the expression

$$\lambda_1 = \frac{M_1 + \sqrt{M_1^2 - 4M_2}}{2}$$

we have

$$\lambda_1 \approx M_1 \quad (10)$$

If we now compare both Eqs. 6 and 10 the result is:

$$\lambda_1 \gg \lambda_2 \quad (11)$$

Otherwise, from Eqs. 7 and 10, we get:

$$\lambda_2 \approx \frac{M_2}{M_1} \quad (12)$$

Inserting Eq. 4 into Eq. 10 and Eqs. 4 and 5 into Eq. 12, in those cases in which the relationship

$$(k_3+k_i)K_4 + k_5K_2 \ll (k_3+k_i)[\mathrm{ROOH}]_0 \quad (13)$$

is yielded, then we have:

$$\lambda_1 \approx \frac{(k_3+k_i)[\mathrm{ROOH}]_0^2}{[\mathrm{ROOH}]_0^2 + (K_2+K_4)[\mathrm{ROOH}]_0 + K_2K_4} \quad (14)$$

$$\lambda_2 \approx \frac{k_ik_5}{k_3+k_i} \quad (15)$$

Note that in those cases in which K_2 is not very much greater than K_4, then, due to the Eq. 8, the relationship:

$$k_5K_2 \ll (k_3+k_i)K_4$$

results, and condition (12) reduces to:

$$K_4 \ll [\mathrm{ROOH}]_0 \quad (16)$$

Inserting relationship (16) into Eq. 14, the latter becomes:

$$\lambda_1 = \frac{(k_3+k_i)[\mathrm{ROOH}]_0}{K_2 + [\mathrm{ROOH}]_0} \quad (17)$$

The existence of a bi-exponential function (linear tracing and non-linear tracing) depending on the incubation time is shown in Fig. 3. Note that at high values of t the plot is linear. This is because of the fact that, due to relationship (11), at high values of t the exponential term

$$\gamma_1 e^{-\lambda_1 t}$$

can be neglected and Eq. 2 simplifies to:

$$\frac{A_t}{A_0} \approx \gamma_2 e^{-\lambda_2 t} \text{ (at high values of t)} \quad (18)$$

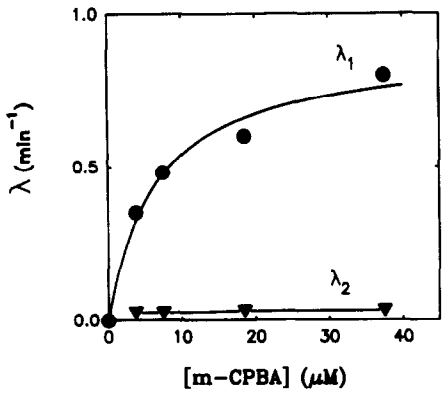


Fig. 4. Dependence of the apparent inactivation constants (●) λ_1 and (▼) λ_2 on the concentration of *m*-CPBA. (—) calculated data using final estimations from non-linear regression analysis.

and, hence:

$$\ln\left(\frac{A_t}{A_0}\right) = \ln\gamma_2 - \lambda_2 t \quad \text{(at high values of t)} \qquad (19)$$

Initial estimates of λ_2 and γ_2 can be obtained from Fig. 3, in the zone showing a linear trace, according to simplified Eq. 19. Once the values of λ_2 and γ_2 have been evaluated, λ_1 and γ_1 may be estimated since, from Eq. 2, we have:

$$\ln\left(\frac{A_t}{A_0} - \gamma_2 e^{-\lambda_2 t}\right) = \ln\gamma_1 - \lambda_1 t \qquad (20)$$

The insert in Fig. 3 shows the values of λ_1 and γ_1 for different *m*-CPBA concentrations.

Thus, a set of λ_1 values is obtained. The non-linear regression of λ_1 vs. [*m*-CPBA] allows us to obtain the values of $(k_3 + k_i)$ and K_2 (Fig. 4). The initial estimates of these parameters are taken from the graphic levels of saturation $(k_3 + k_i)$ and half-saturation (K_2), respectively. From the values of these parameters and from the r value $(r = k_3/k_i)$ (Fig. 1 and Eq. 1), the constants k_3, k_i and K_2 can be calculated. Hence, from the λ_2 values (Fig. 4), k_5 can be evaluated (Eq. 15).

Table 1 presents the kinetic values allowing the characterization of the suicide inactivation of HRP by *m*-CPBA. In summary, if we compare these results with those obtained for H_2O_2 [7]:

1. The values for k_i (kinetic constant for the step [Compound I·ROOH] → E_i) and k_3 (kinetic constant for the step [Compound I·ROOH] → Compound II) are very similar (of the same order of magnitude) for both *m*-CPBA and H_2O_2. However, *m*-CPBA appears as a more powerful inactivator than H_2O_2 since the number of turnovers given by the enzyme before its inactivation (r) is lower with the former. Thus, in the case of *m*-CPBA, it seems that a catalase-like reaction [7] is not active and so the enzyme is not protected.
2. These antecedents suggest that the catalase-like pathway (followed when the enzyme reacts with H_2O_2, but not with *m*-CPBA) contributes more to the r value for H_2O_2 and hence is the main factor responsible for the protection of the enzyme against inactivation. In addition, this low r value for *m*-CPBA is reminiscent of that calculated when HRP, reacting with H_2O_2, follows the Compound III pathway [7].
3. The k_5 constant characterizing the decomposition of the (Compound II·*m*-CPBA) complex is very similar to its equivalent for the (Compound II·H_2O_2) complex, known as Com-

Table 1
Kinetic constants which characterize the suicide inactivation of HRP by *m*-CPBA

Catalytic step	*m*-CPBA	H_2O_2
Compound I + ROOH ⇌ [Compound I·ROOH]	K_2 (M) = (6.54 ± 0.10) · 10^{-6}	K_2(M) = (2.07 ± 0.4) · 10^{-3}
[Compound I·ROOH] → E + O_2	–	k_3 (s^{-1}) = (1.76 ± 0.16)
[Compound I·ROOH] → E_i	k_i (s^{-1}) = (4.80 ± 0.40) · 10^{-3}	k_i (s^{-1}) = (3.92 ± 0.06) · 10^{-3}
[Compound I·ROOH] → Compound II	k_3 (s^{-1}) = (10.08 ± 0.80) · 10^{-3}	k_4 (s^{-1}) = (7.85 ± 0.37) · 10^{-3}
[Compound II·ROOH] → E	k_5 (s^{-1}) = (1.47 ± 0.11) · 10^{-3}	k_7 (s^{-1}) = 2.2 · 10^{-3} (*)
Partition ratio (r)	k_3/k_i = 1.8 ± 0.1	k_4/k_i = 2.00 ± 0.07

The constant value and its unit for each catalytic step, in accordance with Scheme 1, is indicated. Equivalent values for H_2O_2 (taken from reference [7]) are also shown. (*) Value taken from reference [6]

pound III. This coincidence poses questions about the intrinsic nature of both complexes.

4. Also of interest is the low value for K_2 (6.54 μM), indicating a high affinity of Compound I for *m*-CPBA. This quality may be of significance if, across a range of different hydroperoxide structures, those which best form Compound I are also best at inactivating the enzyme. In this sense, it is interesting to remember the reflexion of Dunford and Hewson (1977) [31], about the compromise, developed by peroxidases, between specificity and rate which would protect them from harmful side reactions.

Although it is unlikely that peroxybenzoic acids act as natural oxidizing substrates of the peroxidases, their accumulation as xenobiotics in some polluted environments could affect plant development by altering the normal oxidant/reductant ratio in the tissues. On the other hand, these acids could be useful tools, applied exogenously, to inactivate peroxidases and to follow the consequences in some patterns of plant development where these enzymes are involved, such as growth [32], organogenesis [33] and pathogenesis [34].

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Appendix A. Determination of the partition ratio

The partition ratio (r) corresponds to the number of turnovers given by one mole of enzyme before its inactivation and, according to the mechanism described in Scheme 1, is equivalent to the k_3/k_i ratio.

A relationship between the residual activity of the enzyme, the partition ratio and the substrate/enzyme ratio can be obtained in accordance with [28]. However, it must be taken into account that, in the present case, two molecules of substrate are necessary to induce enzyme inactivation. Hence, following Scheme I, the rates for the product ($ROO^{\cdot}$) and inactive enzyme (E_i) formation are:

$$\frac{d[ROO^{\cdot}]}{dt} = 2k_3[\text{Compound I}\cdot\text{ROOH}] \tag{I.1}$$

$$\frac{d[E_i]}{dt} = k_i[\text{Compound I}\cdot\text{ROOH}]$$

Dividing these previous equations gives:

$$\frac{d[ROO^{\cdot}]}{d[E_i]} = \frac{2k_3}{k_i} \tag{I.2}$$

By integration:

$$\frac{[ROO^{\cdot}]}{[E_i]} = \frac{2k_3}{k_i} \tag{I.3}$$

and

$$r = \frac{k_3}{k_i} = \frac{[ROO^{\cdot}]/2}{[E_i]} \tag{I.4}$$

In the present experimental approach, the substrate is exhausted and the residual activity of the enzyme is determined in such a way that the balance for the substrate is:

$$[ROOH] = [ROH] + [ROO^{\cdot}] + [E_i] \quad (I.5)$$

In accordance with Eq. I.4:

$$[ROO^{\cdot}] = 2r[E_i] \quad (I.6)$$

and

$$[ROH] = \frac{[ROO^{\cdot}]}{2} + [E_i] = (1+r)[E_i] \quad (I.7)$$

By introducing Eqs. I.6 and I.7 in Eq. I.5, gives:

$$[ROOH] = (1+r)[E_i] + 2r[E_i] + [E_i] \quad (I.8)$$

$$[E_i] = \frac{[ROOH]}{3r+2}$$

The active enzyme remaining at the end of the reaction (E_a) is:

$$[E_a] = [E]_0 - [E_i] \quad (I.9)$$

By introducing Eq. I.8 in Eq. I.9 and dividing by $[E]_0$, gives:

$$\frac{[E_a]}{[E]_0} = 1 - \frac{1}{3r+2}\frac{[ROOH]}{[E]_0} \quad (I.10)$$

and, because $[E_a]/[E]_0$ is the residual activity (A_R):

$$A_R = 1 - \frac{1}{3r+2}\frac{[ROOH]}{[E]_0} \quad (I.11)$$

Thus, plotting A_R versus $[ROOH]/[E]_0$ and, according to Eq. I.11, it is possible to calculate the partition ratio (r) for this particular mechanism (Scheme I).

Appendix B. Time course of the peroxidase activity

If the procedure developed by Varón et al. (1990) [30] to obtain the time course of the concentration any species involved in an enzyme reaction in conditions of limiting enzyme is applied to Scheme 1, then, we have:

$$[E_i] = [E]_0 + \sum_{h=1}^{5} A_h e^{\delta_h t} \quad (A1)$$

where $[E]_0$ is the initial concentration of the free enzyme E, $[E_i]$ is the concentration of inactive enzyme, and the meaning of the arguments δ_h ($h = 1, 2, \ldots, 5$) and their amplitudes A_h ($h = 1, 2, \ldots, 5$) are given next.

Arguments δ_h ($h = 1, 2, \ldots, 5$)

Any of the parameters $\delta_1, \delta_2, \ldots, \delta_5$ is negative or complex with a negative real part and they coincide with the zeros of the following polynomial $T(\delta)$:

$$T(\delta) = \sum_{q=0}^{5} F_q \delta^{5-q} = 0 \tag{A2}$$

where:

$$F_0 = 1 \tag{A3}$$

$$F_1 = (k_1 + k_2 + k_4)[S]_0 + k_{-2} + k_3 + k_i + k_5 + k_{-4} \tag{A4}$$

$$F_2 = \{k_1(k_2 + k_4) + k_2 k_4\}[S]_0^2 + \{k_1(k_{-2} + k_3 + k_i + k_5 + k_{-4}) + k_2(k_3 + k_i + k_5 + k_{-4}) + k_4(k_{-2} + k_3 + k_i + k_5)\}[S]_0 + (k_{-4} + k_5)(k_{-2} + k_3 + k_i) \tag{A5}$$

$$F_3 = k_1 k_2 k_4 [S]_0^3 + \{k_1 k_2(k_3 + k_i + k_5 + k_{-4}) + k_1 k_4(k_{-2} + k_3 + k_i + k_5) + k_2 k_4(k_3 + k_i + k_5)\}[S]_0^2 + \{(k_{-4} + k_5)[k_1(k_{-2} + k_3 + k_i) + k_2(k_i + k_3)] + k_4 k_5(k_{-2} + k_3 + k_i)\}[S]_0 \tag{A6}$$

$$F_4 = k_1 k_2 k_4(k_3 + k_5 + k_i)[S]_0^3 + \{k_1 k_2(k_3 + k_i)(k_{-4} + k_5) + k_4 k_5(k_{-2} k_5 + k_3 k_5 + k_1 k_i + k_2 k_3) + k_2 k_i k_4 k_5\}[S]_0^2 \tag{A7}$$

$$F_5 = k_1 k_2 k_4 k_i k_5 [S]_0^3 \tag{A8}$$

$[S]_0$ being the initial concentration of the substrate. From the polynomial theory the following relationships between the above coefficients F_q $(q = 1, 2, \ldots, 5)$ and the roots δ_h $(h = 1, 2, \ldots, 5)$ are obtained:

$$\delta_1 + \delta_2 + \delta_3 + \delta_4 + \delta_5 = -F_1 \tag{A9}$$

$$\delta_1\delta_2 + \delta_1\delta_3 + \ldots + \delta_4\delta_5 = F_2 \tag{A10}$$

$$\delta_1\delta_2\delta_3 + \delta_1\delta_2\delta_4 + \ldots + \delta_3\delta_4\delta_5 = -F_3 \tag{A11}$$

$$\delta_1\delta_2\delta_3\delta_4 + \delta_1\delta_2\delta_3\delta_5 + \delta_1\delta_2\delta_4\delta_5 + \delta_1\delta_3\delta_4\delta_5 + \delta_2\delta_3\delta_4\delta_5 = F_4 \tag{A12}$$

$$\delta_1\delta_2\delta_3\delta_4\delta_5 = -F_5 \tag{A13}$$

Amplitudes A_h $(h = 1, 2, \ldots, 5)$

The expressions for the amplitudes in Eq. (A1) are

$$A_h = \frac{k_1 k_2 k_i(\delta_h^2 + (k_4[S]_0 + k_5 + k_{-4})\delta_h + k_4 k_5 [S]_0 [S]_0^2 [E]_0}{\delta_h \prod_{r=1}^{5} \prod_{r \neq h} (\delta_r - \delta_h)} \quad (h = 1, 2, \ldots, 5) \tag{A14}$$

Next, we consider the particular case in which the following relationships between the rate constants values are observed:

$$k_i, k_3, k_5 \ll k_1[S]_0, k_2[S]_0, k_4[S]_0, k_{-2}, k_{-4} \tag{A15}$$

where

$k_1[S]_0, k_2[S]_0, k_4[S]_0, k_{-2}, k_{-4}$

are not mutually different.

This condition is equivalent to the assumption that the concentration of form E, at any reaction time, is negligible when compared with that of Compound I (because E becomes Compound I immediately) and that the reversible steps are in conditions of rapid equilibrium. Using the magnitude orders of the values of the rate constants k_i, k_3, k_5 as reference points, conditions (A15) are mathematically equivalent to:

$$k_1[S]_0, k_2[S]_0, k_4[S]_0, k_{-2}, k_{-4} \rightarrow \infty \tag{A16}$$

If condition (A16) is now inserted into Eqs. (A3)–(A9), it results in:

$$F_1 = (k_1 + k_2 + k_4)[S]_0 + k_{-2} + k_{-4} \tag{A17}$$

$$F_2 = \{k_1(k_2 + k_4) + k_2k_4\}[S]_0^2 + (k_1k_{-2} + k_1k_{-4} + k_2k_{-4}) + k_4k_{-2})[S]_0 + k_{-4}k_{-2} \tag{A18}$$

$$F_3 = k_1k_2k_4[S]_0^3 + k_1(k_2k_{-4} + k_4k_{-2})[S]_0^2 + k_{-4}k_1k_{-2} \tag{A19}$$

$$F_4 = k_1k_2k_4(k_3 + k_5 + k_i)[S]_0^3 + k_1\{k_2k_{-4}(k_3 + k_i) + k_4k_{-2}k_5\}[S]_0^2 \tag{A20}$$

F_0 and F_5 being unchanged from Eqs. A3 and A8. Note, that from conditions (A16) and Eqs. A3, A17–A20, the following result is observed:

$$\frac{F_i}{F_3} \approx 0 \ (i = 0, 1, 2) \tag{A21}$$

$$\frac{F_4}{F_3} = \frac{(k_3 + k_i + k_5)[S]_0^2 + [(k_3 + k_i)K_4 + k_5K_2][S]_0}{[S]_0^2 + (K_4 + K_2)[S]_0 + K_2K_4} \tag{A22}$$

$$\frac{F_5}{F_3} = \frac{k_ik_5[S]_0^2}{[S]_0^2 + (K_4 + K_2)[S]_0 + K_2K_4} \tag{A23}$$

K_2 and K_4 being k_{-2}/k_2 and k_{-4}/k_4, respectively.

When any polynomial is either multiplied or divided by a constant, the zeros of the resulting polynomial are the same as those in the original one. If we divide polynomial $T(\delta)$ by F_3 it results in the following polynomial $R(\delta)$:

$$R(\delta) = \sum_{q=0}^{5} \frac{F_q}{F_3} \delta^{5-q} \tag{A24}$$

the zeros of which are those of $T(\delta)$. Therefore, the zeros of $T(\delta)$ are the roots of equation:

$$\sum_{q=0}^{5} \frac{F_q}{F_3} \delta^{5-q} = 0 \tag{A25}$$

By finding the finite zeros of $T(\delta)$, the first three terms on the left side of Eq. A22 term of $R(\delta)$ can be neglected if Eq. A21 is taken into account. Therefore, these zeros approximately coincide with the roots of the equation:

$$\delta^2 + M_1\delta + M_2 = 0 \tag{A26}$$

where M_1 and M_2 denote the ratios F_4/F_3 and F_5/F_3, respectively, given by Eqs. A22 and A23.

According to the polynomial theory, between δ_1 and δ_2 the following two relationships are fulfilled:

$$\delta_1 + \delta_2 = -M_1 \tag{A27}$$

and

$$\delta_1\delta_2 = M_2 \tag{A28}$$

The above discussion allows us to state that two of the zeros δ_h ($h=1, 2, \ldots, 5$), let them be δ_1 and δ_2, of polynomial $T(\delta)$ are finite and coincide approximately with the roots of Eq. A26, whereas its other zeros δ_3, δ_4 and δ_5 are, in absolute values, much higher than the absolute values of δ_1 and δ_2, and mathematically can be expressed, using the magnitude orders of δ_1 and δ_2 as reference points, as:

$$\sigma_i \to \infty \quad (i=1, 2, 3) \tag{A29}$$

If relationship (A29) is introduced into Eq. A1, then the three last exponential terms on the right hand side can be neglected and the expressions for the remaining amplitudes given by Eqs. A14 with $h=1$ and 2 can be simplified, so that Eq. A1 becomes:

$$[E_i] = [E]_0 + \sum_{h=1}^{2} A_h e^{\delta_h t} \tag{A30}$$

where δ_1 and δ_2 are the roots of Eq. A26 and A_1 and A_2 are now:

$$A_h = \frac{k_1k_2k_i\{(k_4[S]_0 + k_{-4})\delta_h + k_4k_5[S]_0\}[S]_0^2[E]_0}{\delta_h(\delta_r - \delta_h)\delta_3\delta_4\delta_5} \quad (h=1, 2;\ r=2 \text{ if } h=1 \text{ and } r=1 \text{ if } h=2) \tag{A31}$$

Note, that if relationship (A29) is inserted into Eq. A11, then its left hand side becomes approximately $\delta_3\delta_4\delta_5$ and having taken Eq. A19 into account, it results in:

$$\delta_3\delta_4\delta_5 = -(k_1k_2k_4[S]_0^3 + k_1(k_2k_{-4} + k_4k_{-2})[S]_0^2 + k_{-4}k_1k_{-2}) \tag{A32}$$

Inserting Eq. A32 into Eq. A31 and dividing both numerator and denominator by $k_1k_2k_4$, we obtain:

$$A_h = \frac{k_i\{([S]_0 + K_4)\delta_h + k_5[S]_0\}[S]_0^2[E]_0}{\delta_h(\delta_h - \delta_r)\{[S]_0^2 + (K_2 + K_4)[S]_0 + K_2K_4\}} \quad (h=1, 2;\ r=2 \text{ if } h=1 \text{ and } r=1 \text{ if } h=2) \tag{A33}$$

B.2. Loss in enzyme activity

The loss in the activity of the enzyme can be expressed by the ratio A_t/A_0; A_t and A_0 being the enzyme activity at time t and at $t=0$, respectively. This loss of enzyme activity can be expressed as:

$$\frac{A_t}{A_0} = \frac{[E]_0 - [E_i]}{[E]_0} \tag{A34}$$

and by inserting Eq. A30 into Eq. A34, and setting:

$$\delta_h = -\lambda_h \quad (h=1, 2) \tag{A35}$$

we have:

$$\frac{A_t}{A_0} = \gamma_1 e^{-\lambda_1 t} + \gamma_2 e^{-\lambda_2 t} \tag{A36}$$

γ_h ($h=1, 2$) being:

$$\gamma_h = \frac{k_i\{([S]_0 + K_4)\delta_h + k_5[S]_0\}[S]_0^2}{\delta_h(\delta_r - \delta_h)\{[S]_0^2 + (K_2 + K_4)[S]_0 + K_2K_4\}} \quad (h=1, 2;\ r=2 \text{ if } h=1 \text{ and } r=1 \text{ if } h=2) \tag{A37}$$

Note, that from Eqs. A35, A27 and A28:

$$\lambda_1 + \lambda_2 = M_1 \tag{A38}$$

and

$$\lambda_1 \lambda_2 = M_2 \tag{A39}$$

i.e. γ_1 and γ_2 are the roots of equation:

$$\lambda^2 - M_1 \lambda + M_2 = 0 \tag{A40}$$

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