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Inactivation studies on native and silica gel non-homogeneous immobilized chloroperoxidase

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Abstract

A pH stability analysis was made, in the presence of *tert*-butyl hydroperoxide, of both immobilized and native chloroperoxidase obtained from *Caldariomyces fumago* and the inactivation constants (j_1) evaluated. The native enzyme displays a uni-exponential decay, whereas for the immobilized enzyme a three exponential equation describes the time dependent enzyme inactivation.

For immobilized enzyme, three-exponential equation describes the enzyme time-course inactivation. The obtained inactivation constants (j_3 and K_3) showed an increase in the stability of a fraction of the immobilized enzyme. This is probably due to a decrease of the affinity of the enzyme for the oxidant and not to a decrease in j_3 values.

Keywords: Suicide substrate; Kinetic parameters; Enzyme inactivation; Chloroperoxidase; Time course; Immobilized-enzyme

1. Introduction

Chloroperoxidase from *Caldariomyces fumago* is a versatile and unusual heme-peroxidase. In vitro, chloroperoxidase shows halogenase-, peroxidase-, catalase-activity, and in particular some chloroperoxidase-catalyzed reactions appear to be mediated by cytochrome P450-like mechanisms in which the oxygen of the ferryl ($Fe^{IV}=O$) species, which derives from H_2O_2, is transferred to the substrate [1]. The mammalian cytochrome P450 monooxygenases catalyze the metabolism of a wide variety of endogenous and exogenous compounds, including steroids, therapeutic drugs, and carcinogens [2]. In some cases, the formation of a reactive intermediate by P450 may also lead to the inactivation of the enzyme. P450 substrates, which are metabolized to reactive intermediates that inactivate the enzyme, are classified as mechanism-based inactivators [3]. Mechanism-based inactivation of P450 enzymes involves metabolic activation of the substrate followed by binding of a reactive intermediate to either the heme- or the apo-protein to render the enzyme inactive [4]. So, in several works there are cytochrome P450 time- and concentration-dependent inactivation studies that reflect the suicide nature of several P450 substrates.

The progress curve for the irreversible product accumulation will be described by integrated Michaelis–Menten equation [5], in which the dependence of [S] on time is implicit. If the substrate is a suicide one, the enzyme suffers a loss of activity during the incubation. Waley [6,7] proposed implicit integrated equations which describe either the consumption of substrate or the loss of enzyme activity. From mechanism discussed by Waley it was successively derived [8] explicit time-course equations for the accumulation of the product assuming that the substrate concentration remains approximately constant during the period considered. That condition can be achieved experimentally using a suitable value of the enzyme concentration. The experimental design has been applied to the kinetic study of the inactivation of tyrosinase by various suicide substrates [9,10].

Duggleby has made an important theoretical contribution to the problem of enzyme systems in which one or more of the enzyme forms are unstable [11]. The approach is based upon measurement of progress curves of the substrate utilisation. Duggleby suggests a graphical method, the *J* plot, for the

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determination of the inactivation rate constants from the final substrate concentration.

Kinetic analysis has been made for the case in which the free enzyme, or the enzyme–substrate complex, or both, are unstable, either spontaneously or as a result of the addition of a suicide substrate. The explicit time-course equations of all of the species involved have been derived [12–14] assuming that the substrate concentration remains approximately constant during the period considered. That condition can be achieved experimentally using a suitable value of the enzyme concentration. However, it is well known that when an enzyme able to catalyse an irreversible two substrates/two products reaction is incubated with only one substrate, the total concentration of the substrate can be considered as a constant. In our case the only substrate used is the oxidant whose concentration will be constant and we study the enzyme inactivation.

Interest in these systems stems not only from their obvious biological importance but also from a desire to harness their synthetic potential. Because alkane hydroxylation and olefin epoxidation are important industrial reactions, engineered immobilized enzymes that could efficiently activate and transfer oxygen would be of considerable economic value [15]. In comparison with their native form, immobilized enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of continuous processes and repetitive enzyme use. However, in the covalent immobilisation, an enzyme suffers a loss of homogeneity: each appeared enzyme population type shows different half-life after incubation in a simple pH buffer solution. Moreover, a suicide substrate is present, the problems enormously increase.

The aim of this paper is to derive the time-course equation of the oxidant inactivated species under assumptions mentioned in theory and by using these kinetic analyses to study the inactivation of the silica-immobilized chloroperoxidase in the presence of the oxidant *tert*-butyl hydroperoxide.

2. Materials and methods

2.1. Experimental

2.1.1. Materials

2.1.1.1. Reagents. Silica gel (5 μm, specific surface of 340 m^2/g) was from Alltech. (3-Glycidyloxypropyl)trimethoxysilane was supplied by Fluka. Chloroperoxidase (CPO) from *C. fumago* (chloride hydrogen-peroxide oxidoreductase [EC 1.11.1.10]) and *tert*-butyl hydroperoxide were supplied by Fluka and were used without further purification. Monochlorodimedone was a Sigma Chemical Co. product and was used without further purification. All other reagents were commercial products of analytical grade. UV and visible spectrophotometric measurements were performed at room temperature on Varian CARY 04-E spectrophotometer interfaced to a personal computer.

2.1.1.2. Preparation of immobilized chloroperoxidase. Epoxide derivatized silica gel was prepared according to the methods reported [16]. Four milliliters of 0.05 M potassium phosphate buffer solution, pH 6, containing ammonium sulphate 2 M and 250 μl of CPO suspension (12.6 mg/ml) were added to 0.5 g of functionalised silica gel. The mixture was stirred at room temperature for 2 h. The solid with the immobilized enzyme was then recovered through centrifugation and the UV spectrum of the supernatant was recorded at 400 nm. The recovered solid was washed with the same buffer (0.05 M, pH 6) until the disappearing of the maximum at 400 nm in the UV spectrum ($\varepsilon_{400} = 91\ mM^{-1}\ cm^{-1}$). The amount of enzyme bound to the support (2.71 mg CPO/g of silica gel) was calculated from the UV absorbance by subtracting the absorbance of the supernatant plus washings from the initial absorbance of the enzyme solution. The experiment was carried out in duplicate. The SiO_2–CPO preparations were stored at −20 °C until use.

2.1.2. Methods

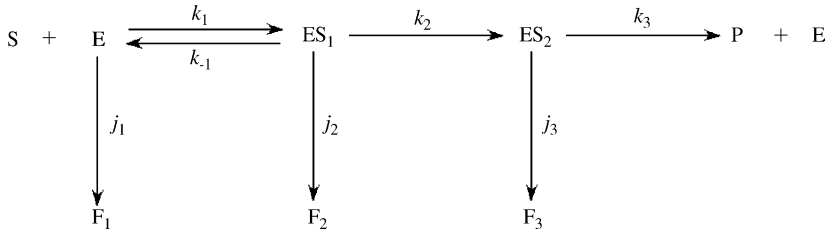
2.1.2.1. Native chloroperoxidase stability studies. The stability of chloroperoxidase in the presence or the absence of the oxidant, *tert*-BuOOH, was investigated. Ten microlitres of Fluka commercial enzyme were pre-incubated in 10 ml of 0.1 M potassium phosphate buffers of five different pH values (pH 2.75–7). The pre-incubations were performed in triplicates. Ten microlitres of Fluka commercial enzyme were pre-incubated with different concentrations of the oxidant (0.1–7.3 mM) in 10 ml of 0.1 M potassium phosphate buffer (pH 6). The pre-incubations were performed in duplicates with three data point per concentration.

During all pre-incubations, the tubes were shaken at 200 rpm by using an orbital shaker.

2.1.2.2. Silica–chloroperoxidase stability studies. The stability of immobilized-chloroperoxidase in the presence or the absence of the oxidant *tert*-BuOOH was investigated. Ten milligrams of immobilized enzyme were pre-incubated in 10 ml of potassium phosphate buffers (0.1 M) at five different pH values (pH 2.75–7). The pre-incubations were performed in triplicates. Ten milligrams of immobilized enzyme were pre-incubated with different concentrations of the oxidant (0.1–7 mM) in 10 ml of 0.1 M potassium phosphate buffer (pH 6.00). The pre-incubations were performed in duplicates with three data point per concentration.

During all pre-incubations, the tubes were shaken at 200 rev/min by using an orbital shaker.

2.1.2.3. Spectrophotometric assay. A fixed amount (200 μl) of pre-incubation solutions (native, 2.2 U/ml; immobilized, 2 mg/ml) was taken (from 0 to 10^6 s) and added to a standard reaction mixture, consisting of potassium chloride (0.11 mmol), *tert*-butyl hydroperoxide (0.2 mmol), monochlorodimedone (MCD, 0.27 μmol) in 0.1 M potassium phosphate buffer (pH 2.75) in a total volume of 5.4 ml. The reaction was followed by monitoring the decrease in absorbance at 278 nm due to the conversion of monochlorodimedone ($\varepsilon_{278} = 1.22 \times 10^4$) to dichlorodimedone (DCD, $\varepsilon_{278} = 1.6 \times 10^2$) [17].



Scheme 1. Inactivation scheme of an enzyme in the presence of suicide substrate.

3. Theory

3.1. Kinetic analysis

Garrido-del Solo et al. [12] and Toti et al. [18] have presented a kinetic analysis of a Michaelis–Menten mechanism in which the enzyme is unstable. We considered the case in which enzyme E binds to a molecule S forming a first intermediate (ES_1); then complex ES_1 is transformed in complex ES_2. The case in which the enzyme specifically binds to S (oxidant), and either the free enzyme E or the enzyme-substrate complexes ES_1 and ES_2 are unstable, can be described by Scheme 1. Note that we use a two intermediate scheme because we have verified that for native form at least two intermediates appeared [18]; the simplification by using a single intermediate scheme leads to equations more simple but not close to experimental system. Moreover, the time-course solutions could be used, by an algebraic artifice with the use of γ functions (see Eqs. (18)–(20)), also in the cases of two or three exponential behaviour.

In this contribution we analyze the kinetics of m enzyme reactions evolving according to Scheme 2, where S is a suicide substrate, assuming that (from $n = 1$ to m) j_{3n} inactivation constant is much higher than the k_{3n} catalytic constant and that the substrate concentration remains approximately constant during the assay time considered. The latter assumption can be achieved experimentally using an initial value of the enzyme concentration that is small enough compared with the initial substrate concentration. Suicide substrates may be defined in terms of their dual role as compounds that interact with enzymes by a mechanism with a branched pathway, the branches representing turnover of the substrate and inactivation of the enzyme [18–20].

We extend now this scheme and its solutions on a non-homogeneous enzyme population system, in which m enzyme populations are independent each other (Scheme 2). In Scheme 2, E_n is the nth free enzyme species, S the substrate, ES_{1n} and ES_{2n} the nth intermediate species and F_{1n}, F_{2n} and F_{3n} are the nth inactive species into which the species E_n, ES_{1n} and ES_{2n}, respectively, are transformed.

The equations describing the kinetics of the species in Scheme 2 are

$$\frac{d[E_n]}{dt} = -(k_{1n}[S] + j_{1n})[E_n] + k_{-1n}[ES_{1n}] + k_{3n}[ES_{2n}] \quad (1)$$

$$\frac{d[ES_{1n}]}{dt} = k_{1n}[E_n][S] - (k_{-1n} + j_{2n} + k_{2n})[ES_{1n}] \quad (2)$$

$$\frac{d[ES_{2n}]}{dt} = k_{2n}[ES_{1n}] - (j_{3n} + k_{3n})[ES_{2n}] \quad (3)$$

$$\frac{d[F_{1n}]}{dt} = j_{1n}[E_n] \quad (4)$$

$$\frac{d[F_{2n}]}{dt} = j_{2n}[ES_{1n}] \quad (5)$$

$$\frac{d[F_{3n}]}{dt} = j_{3n}[ES_{2n}] \quad (6)$$

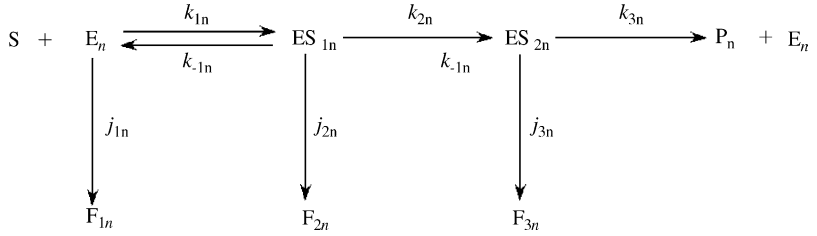
$$\frac{d[P_n]}{dt} = k_{3n}[ES_{2n}] \quad (7)$$

$$\frac{d[S]}{dt} = -k_1[E_n][S] + (k_{-1n})[ES_{1n}] \quad (8)$$

and the two mass balance are

$$[E]_0 = \sum_{n=1}^{m}([E_n] + [ES_{1n}] + [ES_{2n}] + [F_{1n}] + [F_{2n}] + [F_{3n}]) \quad (9)$$

$$[S]_0 = [S] + \sum_{n=1}^{m}([ES_{1n}] + [ES_{2n}] + [P_n]) \quad (10)$$



Scheme 2. Inactivation scheme of nth enzyme in the presence of suicide substrate.

The set of differential equations (1)–(8) is non-linear and has, therefore, no analytical solution. However, under assumptions (11) and (12) extended for m populations:

$$[E_n]_0 \ll [S]_0 \tag{11}$$

$$k_{3n} \ll j_{3n} \tag{12}$$

Eq. (10) simplifies to

$$[S]_0 \approx [S] \tag{13}$$

Assumption (12) is consistent with the extremely low utilisation, by native CPO, of the substrate *t*BuOOH [21]; this assumption was verified by us in the free enzyme study [18] in which the enzyme inactivation was a perfect uni-exponential type. The product formation could be lead an eviden distortion in the exponential behaviour.

Upon insertion of relation (13) into Eqs. (1)–(7), the set becomes linear. The solution for F_{3n} species is (14):

$$[F_{3n}] = \frac{j_{3n}k_{2n}k_{1n}[E_n]_0[S]_0}{G_{3n}} + \sum_{h=1}^{3} \frac{j_{3n}k_{2n}k_{1n}[E_n]_0[S]_0}{\gamma_{hn} \prod_{\substack{p=1 \\ p\neq h}}^{3} (\gamma_{pn} - \gamma_{hn})} e^{\gamma_{hn}t} \tag{14}$$

where γ_{1n}, γ_{2n} and γ_{3n} are the nth roots of the nth polynomial $\gamma^3 + G_{1n}\gamma^2 + G_{2n}\gamma + G_{3n}$ relatives to nth specie in which

$$G_{1n} = k_{1n}[S]_0 + j_{1n} + j_{2n} + j_{3n} + k_{-1n} + k_{2n} + k_{3n} \tag{15}$$

$$G_{2n} = (j_{2n} + k_{-1n} + k_{2n})(j_{3n} + k_{3n}) + k_{1n}[S]_0(j_{2n} + j_{3n} + k_{2n} + k_{3n}) + j_{1n}(j_{2n} + j_{3n} + k_{-1n} + k_{2n} + k_{3n}) \tag{16}$$

$$G_{3n} = k_{1n}[S]_0(j_{2n}j_{3n} + j_{2n}k_{3n} + k_{2n}j_{3n}) + j_{1n}(j_{2n} + k_{-1n} + k_{2n})(j_{3n} + k_{3n}) \tag{17}$$

Note that γ_{1n}, γ_{2n} and γ_{3n} are all negative terms, and that the following relations exist between the roots:

$$\gamma_{1n} + \gamma_{2n} + \gamma_{3n} = -G_{1n} \tag{18}$$

$$\gamma_{1n}\gamma_{2n} + \gamma_{2n}\gamma_{3n} + \gamma_{1n}\gamma_{3n} = G_{2n} \tag{19}$$

$$\gamma_{1n}\gamma_{2n}\gamma_{3n} = -G_{3n} \tag{20}$$

3.2. Stability studies

In a single experimental procedure it is very hard to obtain for all the m populations both j_1 and j_3 values. So it is necessary to find separately the two inactivation rate constants. We projected two incubation studies, with or without substrate.

For the calculus of j_1 we impose in Scheme 2 that there is no substrate; the set of analytical solutions becomes for the nth population:

$$[E_n] = [E_n]_0 e^{-j_{1n}t} \tag{21}$$

$$[F_{1n}] = [E_n]_0(1 - e^{-j_{1n}t}) \tag{22}$$

For the nth species the enzyme activity units ($EA_{(n)}$) are proportional to the active nth enzyme concentration; so at zero time there are maximal enzyme activity units ($EA_{(n)0}$) in relation with $[E_n]_0$:

$$[E_n] = [E_n]_0 \frac{EA_{(n)}}{EA_{(n)0}} \tag{23}$$

For the nth species, it is possible to express the nth free active enzyme concentration as a function of the nth total enzyme concentration, the $EA_{(n)}$ at t time and the $EA_{(n)0}$. Extending the summation to the m species we obtain Eq. (24):

$$\sum_{n=1}^{m} EA_{(n)} = \sum_{n=1}^{m} (EA_{(n)0} e^{-j_{1n}t}) \tag{24}$$

in which $\sum_{n=1}^{m} EA_{(n)}$ is the enzyme activity units number (EA) detectable in the inactivation experiments. For the nth species either the j_{1n} rate constant or the $EA_{(n)}$ number can be obtained by fitting the results of the enzyme incubation in different pH buffer solutions on a multiexponential fitting method [22,23].

The native enzyme (not bound to silica gel) is a homogeneous population so $m = 1$; we obtain from Eq. (24) a simple first order decay. We fit this equation on a semi-logarithmic plot [24].

By dividing Eq. (24) for the total enzyme activity units number (EA_0), we obtain

$$\frac{EA}{EA_0} = \sum_{n=1}^{m} (f_{(n)} e^{-j_{1n}t}) \tag{25}$$

in which $f_{(n)}$ is the nth fraction of total enzyme activity units number:

$$f_{(n)} = \frac{EA_{(n)0}}{EA_0} \tag{26}$$

For the calculus of j_3 and K_3 [see text] values for m populations it is necessary the presence of the suicide substrate; that is, the study of the general analytical solutions is very difficult. But for the nth species in many cases of practical interest (mono-exponential behaviour):

$$-\gamma_{1n}, -\gamma_{2n} \gg -\gamma_{3n} \tag{27}$$

Eq. (27) becomes true if relations (28) [20] and (29) are verified:

$$j_{1n}, j_{2n}, j_{3n} \ll k_{1n}[S]_0, k_{-1n}, k_{2n} \tag{28}$$

$$j_{3n} \gg j_{1n}, \ j_{2n}, k_{3n} \tag{29}$$

It follows that

$$\gamma_{1n} + \gamma_{2n} = -G_{1n} \tag{30}$$

$$\gamma_{1n}\gamma_{2n} = G_{2n} \tag{31}$$

$$\gamma_{3n} = -\frac{G_{3n}}{G_{2n}} \tag{32}$$

and so γ_{3n} is

$$-\gamma_{3n} = \frac{j_{1n}(j_{2n} + k_{-1n} + k_{2n})(j_{3n} + k_{-3n}) + k_{1n}(j_{2n}j_{3n} + j_{2n}k_{3n} + k_{2n}j_{3n})[S]_0}{j_{1n}(j_{2n} + k_{-1n} + k_{2n} + j_{3n} + k_{3n}) + (k_{3n} + j_{3n})(j_{2n} + k_{-1n} + k_{2n}) + k_{1n}(j_{2n} + j_{3n} + k_{2n} + k_{3n})[S]_0} \tag{33}$$

from Eq. (33) and with conditions (28), (29) and (12) we obtain (34) that is an extension of Eq. (57) of [18]:

$$-\gamma_{3n} = \frac{j_{3n}[S]_0}{\frac{(j_{1n}+j_{3n})(k_{-1n}+k_{2n})}{k_{1n}k_{2n}} + [S]_0} \tag{34}$$

if we express a new constant as $K_{3n} = (j_{1n} + j_{3n})(k_{-1n} + k_{2n})/(k_{1n}k_{2n})$, we obtain (35):

$$-\gamma_{3n} = \frac{j_{3n}[S]_0}{K_{3n} + [S]_0} \tag{35}$$

Note that K_{3n} represents the half rate reaction concentration value and so (35) we had obtained a Michaelis–Menten similar equation. Using relations (28) and (29) allow one to neglect the terms involving $e^{\gamma_{1n}t}$ and $e^{\gamma_{2n}t}$ in the analytical solutions, and with Eqs. (14), (17) and (35) we obtain Eq. (36):

$$[F_{3n}] = [E_n]_0(1 - e^{-((j_{3n}[S]_0)/(K_{3n}+[S]_0))t}) \tag{36}$$

and its derivative equation (37):

$$\frac{d[F_{3n}]}{dt} = [E_n]_0 \frac{j_{3n}[S]_0}{K_{3n} + [S]_0} e^{-((j_{3n}[S]_0)/(K_{3n}+[S]_0))t} \tag{37}$$

Note that for the nth species the $[F_{1n}]$ and $[F_{2n}]$ values are always negligible with respect to all other concentration values.

From the above-discussed limitations and deriving mass balance for the enzyme species, Eq. (9), we obtain Eq. (38). Eq. (38) represents a sort of pseudo steady state between different enzyme forms and the inactive enzyme F_{3n} molecule:

$$\frac{d[F_{3n}]}{dt} = -\frac{d[E_n]}{dt} - \frac{d[ES_{1n}]}{dt} - \frac{d[ES_{2n}]}{dt} \tag{38}$$

The enzyme activity units of the nth species, $EA_{(n)}$, are proportional to the sum of the nth enzyme active species concentrations (free and bound); at zero time there are maximal enzyme activity units, $EA_{(n)0}$, in relation with $[E_n]_0$. So the first derivative of $[F_{3n}]$ species is

$$\frac{d[F_{3n}]}{dt} = -[E_n]_0 \frac{d}{dt}\left(\frac{EA_{(n)}}{EA_{(n)0}}\right) \tag{39}$$

By using Eqs. (26), (38) and (39) and multiplying all the terms by $f_{(n)}$, we obtain

$$v_{(n)} = -\frac{d}{dt}\left(\frac{EA_{(n)}}{EA_0}\right) = \frac{f_{(n)}j_{3n}[S]_0}{[S]_0 + K_{3n}} e^{-((j_{3n}[S]_0)/([S]_0+K_{3n}))t} \tag{40}$$

For the nth species we had to define $v_{(n)}$, expressed in terms of time^{-1}, as ratio of the reaction rate (either of formation of F_{3n} species or of consumption of the three species E_n, ES_{1n} and ES_{2n}) to $[E]_0$; v_n is obviously related to the different peroxide concentrations and time.

At zero time:

$$v_{(n)0} = \frac{f_{(n)}j_{3n}[S]_0}{[S]_0 + K_{3n}} \tag{41}$$

Extending the summation to the m species we obtain Eq. (42):

$$v_0 = \sum_{n=1}^{m} v_{(n)0} = \sum_{n=1}^{m}\left(\frac{f_{(n)}j_{3n}[S]_0}{[S]_0 + K_{3n}}\right) \tag{42}$$

in which we define $\sum_{n=1}^{m} v_{(n)0}$, the ratio of the summation of all initial reaction rate (either of formation of all F_{3n} species or of consumption of all the species E_n, ES_{1n} and ES_{2n}) to $[E]_0$, as v_0.

We can obtain v_0 measuring the initial slope of the plot EA versus time; we assume that this not single exponential function at zero time is a straight line.

For the m species either the fj_3 rate constants or the K_3 values can be calculated by a non-linear fitting. Note that, for the nth species, only by using the $f_{(n)}$ value we can obtain the true j_{3n} value. For the native enzyme, from Eq. (42) by imposing $m = 1$ (and so $f_{(1)} = 1$) we obtain a simple rectangular hyperbola; we can fit this equation by using a double reciprocal plot [25].

If we determine three enzyme population types ($m = 3$), and two of the bound enzymes types present have similar K_3 and j_3 values ($K_{31} \approx K_{32}$ and $j_{31} \approx j_{32}$); we can obtain Eq. (43):

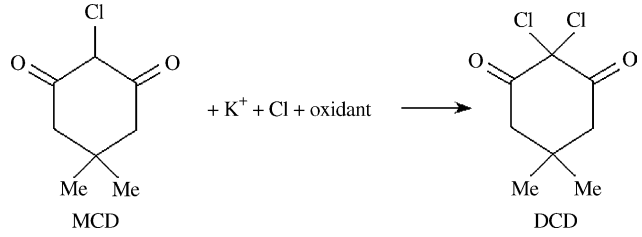
$$v_0 = \frac{(f_{(1)} + f_{(2)})j_{31}[S]_0}{[S]_0 + K_{31}} + \frac{f_{(3)}j_{33}[S]_0}{[S]_0 + K_{33}} \tag{43}$$

where $(f_{(1)} + f_{(2)})$ is the fraction of total enzyme present in the population type $m = 1$ and 2.

4. Results and discussion

The first step in the analysis is to discriminate the amounts of each enzyme population type. The second step is to test the behaviour of the silica-immobilized chloroperoxidase in the presence of inactivating suicide substrate by measuring (for nth populations) either enzyme population type inactivation constants j_{3n} or enzyme population type K_{3n} constants.

The denaturation constants can be measured with a method based on the loss of enzyme activity, using the standard assay involving the monochlorodimedone, chloride and an oxidant at pH 2.75 [22] (Scheme 3). *Tert*-butyl hydroperoxide (*tert*-



Scheme 3. Conversion of monochlorodimedone to dichlorodimedone catalyzed by chloroperoxidase.

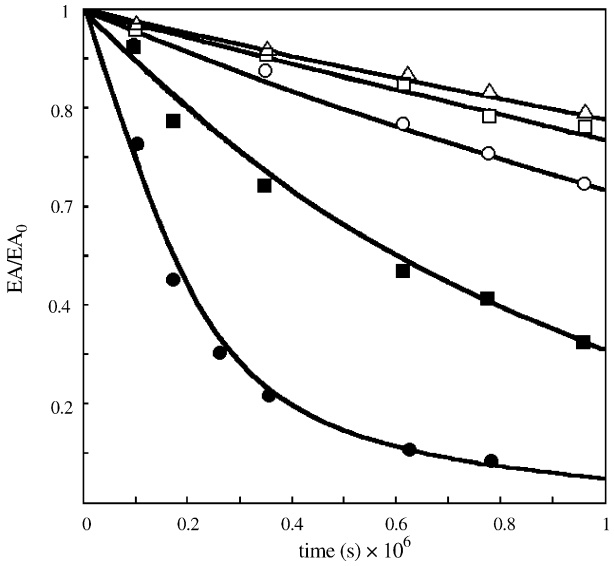


Fig. 1. (a) Native enzyme activity first order decay in buffer potassium phosphate 0.1 M: pH 2.75 (■), pH 4 (◯), pH 5 (□), pH 6 (△) and pH 7 (●). Data were fitted by using the MATLAB programs pocket (polifit.m).

Table 1
Inactivation constants of native chloroperoxidase at various pH values

pH	j_1 (s^{-1})
2.75	$1.08(\pm0.04)\times10^{-6}$
4	$4.46(\pm0.08)\times10^{-7}$
5	$3.24(\pm0.14)\times10^{-7}$
6	$2.42(\pm0.03)\times10^{-7}$
7	$3.61(\pm0.19)\times10^{-6}$

BuOOH) was chosen because with this oxidant the enzyme has low catalase activity [21].

As is shown in Fig. 1 the incubation of native chloroperoxidase with potassium phosphate buffers of different pH values leads to a first order decay of the enzyme activity relative to time. The j_1 values obtained from these experimental data and expressed in s^{-1} are reported in Table 1.

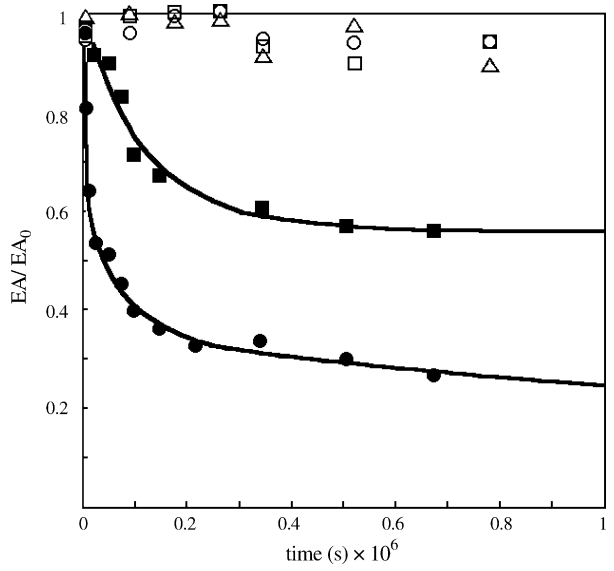


Fig. 2. (a) Silica–enzyme gel activity non-first order decay in buffer potassium phosphate 0.1 M: pH 2.75 (■), pH 4 (◯), pH 5 (□), pH 6 (△) and pH 7 (●). Data were fitted by using the MATLAB programs pocket (mexpfit.m).

Table 2
Inactivation constants of immobilized chloroperoxidase at various pH values

	Type	pH 7
j_{1n} (s^{-1})	1	$8.08(\pm2.22)\times10^{-8}$
	2	$4.66(\pm0.52)\times10^{-6}$
	3	$4.25(\pm0.11)\times10^{-4}$
$EC_{(n)0}$ ($M\,s^{-1}$)	1	$1.19(\pm0.03)\times10^{-2}$
	2	$1.06(\pm0.09)\times10^{-2}$
	3	$1.27(\pm0.04)\times10^{-2}$
$f_{(n)}$	1	0.338(±0.018)
	2	0.301(±0.017)
	3	0.360(±0.001)
	Type	pH 2.75
j_{1n} (s^{-1})	l or 2	$1.02(\pm0.09)\times10^{-7}$
	3	$3.39(\pm1.08)\times10^{-6}$
$EC_{(n)0}$ ($M\,s^{-1}$)	1 + 2	$2.35(\pm0.47)\times10^{-2}$
	3	$1.25(\pm0.16)\times10^{-2}$
$f_{(n)}$	1 + 2	0.645(±0.076)
	3	0.354(±0.076)

In contrast to the native enzyme, the incubation of the immobilized chloroperoxidase, under the same experimental conditions, does not lead to a first order decay of activity (Fig. 2). As can be observed in Fig. 2, there is a non-first order decay of the enzyme activity at pH 2.75 and 7. At the intermediate pH values, however, the immobilized enzyme system shows a high stability and the evaluation of the j_1 constants is not possible.

We remember at this point that the binding of the enzyme to derivatized silica is of covalent type and it can account for possible activity variation [16,26].

By using the experimental data at pH 2.75 and 7, it was possible to distinguish three enzyme population types. This non-homogeneity could reflect the non-homogeneity of the enzyme sample, but it is not so because the decay of native enzyme sample is mono-exponential. The j_1 constants and the fractions of total enzyme activity units $f_{(1)}$, $f_{(2)}$ and $f_{(3)}$ for these enzyme populations are reported in Table 2. The loss of enzyme activity (ca. 5%) observed at pH 4–6, nevertheless it could represent a tendency line, seems to be due rather to experimental error.

Thus, from these data it is clear that immobilized enzyme is more stable, under our experimental conditions, than the native enzyme. Only the population 3 type shows a large decrease in activity at pH 7 (100-fold). The population 3 type at pH 2.75 and population 2 type at pH 7 show a very little decrease.

Table 3
j_3 and K_3 values of native chloroperoxidase (pH 6)

	CPO native
j_3 ($\times10^{-4}\,s^{-1}$)	4.51(±0.21)
K_3 ($\times10^{-3}$ M)	6.02(±0.24)

Table 4
$f_{(n)}$, j_3 corrected and K_3 values of immobilized chloroperoxidase (pH 6)

Silica–CPO	$f_{(n)}$	$f_{(n)}j_{3n}$ ($\times 10^{-4}\,s^{-1}$)	Corrected j_{3n} ($\times 10^{-4}\,s^{-1}$)	K_{3n}
Type 1 + 2	0.643(±0.09)	6.22(±0.45)	9.67(±l.52)	$4.05(\pm0.29)\times10^{-2}$
Type 3	0.357(±0.07)	2.59(±0.24)	7.25(±1.57)	$4.59(\pm0.41)\times10^{-3}$

In addition, we evaluated the effect of different concentrations of the oxidant, *tert*-butyl hydroperoxide, on the stability at pH 6 either of the native enzyme or of the immobilized chloroperoxidase. Using the double reciprocal plot (Lineweaver and Burk) [25] the values of j_3 and the K_3 obtained were $4.51(\pm0.21)\times10^{-4}\,s^{-1}$ and $6.02(\pm0.24)\times10^{-3}$ M, respectively (Table 3).

In Fig. 3 we can show the relationship between v_0 versus the oxidant *tert*-butyl hydroperoxide for the immobilized enzyme as a sum of 1–3 population type.

Table 4 reports the corrected j_3 and K_3 values, obtained from immobilized enzyme. The correction of j_3 values consists in the use of the mean of f values obtained from Table 2. Further, we assume that j_{31} and j_{32} are equal. The correction is necessary because we studied the initial inactivation rate and not directly the exponential terms. This is for the problem in maintaining constant the oxidant concentration in the experimental system we built up.

It is concluded that, in terms of j_3, the absolute stability of the immobilized CPO is similar to that of the native enzyme (j_{31} and j_{33} were $9.67(\pm1.52)\times10^{-4}$ and $7.25(\pm1.57)\times10^{-4}\,s^{-1}$, respectively), whereas its relative stability in the presence of the oxidant was probably increased due to loss of affinity of the enzyme for the oxidant, as suggested by the high K_3 value (Table 4). The K_3 value for two enzyme population type (K_{31} or K_{32} is almost equal to $4.05(\pm0.29)\times10^{-2}$ M) was found to be ten times higher than those either of the third population type (K_{33} as $4.59(\pm0.41)\times10^{-3}$ M) or of the native chloroperoxidase.

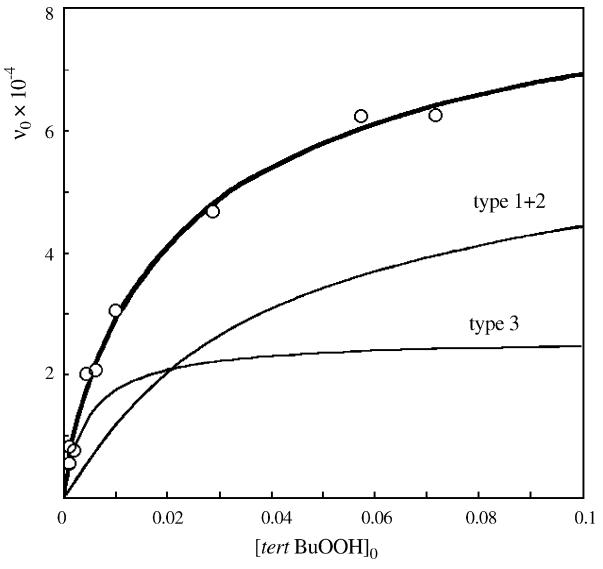


Fig. 3. Relationship between v_0 vs. *tert*-butyl hydroperoxide. The thin line shows the contributes of enzyme type 3 and type 1 + 2. Data were fitted by using the MATLAB programs pocket (curvefit.m).

5. Conclusions

By using general Scheme 2, a kinetic analysis and an experimental procedure for the *n*th population kinetic parameters evaluation have been presented. It is based upon the enzyme inactivation with or without suicide substrate under *m* conditions:

$$[E_n]_0 \ll [S]_0 \quad (\text{with } n = 1, 2, \ldots, m)$$

Depending on relative values of the *n*th rate constants or substrate concentration, the time course of the *n*th population enzyme type inactivation is a tri-, bi-, uni-exponential equation. Overall a non-homogeneous enzyme system has *m* different enzyme population types that lead to a multi-exponential equation (so the maximum number of exponential terms was given by 3*m*). It was proposed an experimental procedure to obtain for each *n*th population the j_{1n}, j_{3n} and K_{3n}; no other microscopic constants can be evaluated.

We have immobilized chloroperoxidase to silica gel in order to increase its stability either in buffer solution or in the presence of the oxidant *tert*-butyl hydroperoxide. The binding between enzyme and silica gel resulted in a non-homogeneous enzyme population. By studying the loss of the silica–enzyme gel activity at pH 2.75 and 7, it was found the j_1 values of three different enzyme populations. Two populations of the immobilized enzyme showed an apparent increase in the stability both to the pH or to the presence of the oxidant, but the latter seems to be due to a decrease of enzyme affinity for the oxidant rather than a decrease of j_3 values, those remained close to the native enzyme value. This non-homogeneity of the enzyme after binding in not due to non-homogeneity of the native enzyme sample, because its decay was found to be mono-exponential.

This strategy to bind enzyme to silica gel, in order to simplify the enzyme recovery after a reaction use, produced two enzyme type populations extremely resistant to pH and to oxidant *tert*BuOOH inactivation.

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