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Polymer 41 (2000) 8183–8192

polymer

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Enzyme-mediated initiation of acrylamide polymerization: reaction mechanism

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Received 29 December 1999; received in revised form 29 February 2000; accepted 18 March 2000

Abstract

The polymerization of acrylamide in water initiated by a horseradish peroxidase-catalyzed redox system is studied. It combines hydrogen peroxide (as the oxidant) and 2,4-pentanedione (as the reductant). Several side reactions leading to the degradation of the enzyme through the formation of compound III are evidenced by polymerization experiments and UV spectroscopy; they can be avoided by adjusting the reactant concentrations. The oxidation of 2,4-pentanedione by a non-enzymic pathway is also detected by ^{1}H NMR. Its main effect is to reduce the concentration of the enol form of 2,4-pentanedione, and hence to modify the initiation rate. From this information, a schematic picture of the reactions involved in the enzyme-catalyzed redox system is drawn, so as to optimize the polymerization conditions.

Keywords: Oxidoreductase; Horseradish peroxidase; Free-radical polymerization

1. Introduction

Several enzyme-catalyzed oxidoreductions are known to generate free-radicals as intermediates [1]. These species are potential initiators for the polymerization of vinyl monomers, for instance. A well-known example of such enzymes is horseradish peroxidase (HRP) which catalyzes the oxidation of several organic substances by hydrogen peroxide (H_2O_2) and a few hydroperoxides [2]. The general mechanism is depicted in Scheme 1 (commonly accepted biocatalytic cycle of horseradish peroxidase; the porphyrin ring is schematically drawn by the two horizontal lines [2,3,20]) and involves the production of free radicals [3,4]. This property has been widely used in the case where phenol is the reducing species, leading to new families of polyphenols [5–8].

As for vinyl monomers, HRP-mediated polymerization was first reported by Derango et al. with acrylic monomers such as acrylamide and hydroxyethyl methacrylate [9]. Nevertheless, the precise mechanism was not investigated and the authors assumed the oxidized form of the enzyme to be the initiator. The HRP/H_2O_2 system was extended to the chemoselective polymerization of 2-(4-hydroxyphenyl) ethyl methacrylate [10]. More recently, laccase was found to induce the acrylamide polymerization in water without an additive at temperatures ranging from 50 to 80°C [11]; however, at room temperature, the addition of a β-diketone is needed to efficiently initiate the polymerization. In this respect, Kobayashi's studies confirmed our previously published works on the key role of β-diketones as mediators for free-radical polymerization by oxidoreductase catalysis at room temperature.

Since 1995, our laboratory has investigated the HRP-mediated polymerization of acrylamide using β-diketones as the reducing substrates [12–14]. Due to specific stabilizations, the enol content of β-diketones is generally much higher than that of ketones [15]. This peculiar property makes β-diketones suitable substrates for HRP since it is known that only the enol form of carbonyl compounds reacts with the enzyme [16].

For instance, malonaldehyde has been shown to react with HRP while having one functional group completely enolized in aqueous solution [17]. In this respect, 2,4-pentanedione (acetylacetone, Acac) was particularly used in our laboratory and it has been shown that the ternary system (HRP/H_2O_2/Acac) readily initiated the polymerization of acrylamide [12–14].

In such enzymatic polymerizations, we should separate

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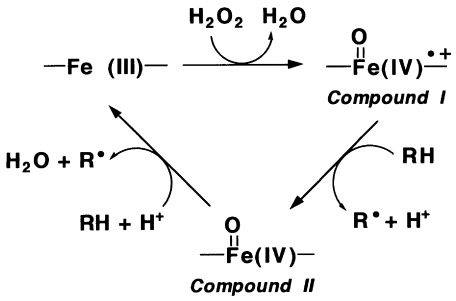
PII: S0032-3861(00)00204-4



Scheme 1.

the elementary steps into two parts: (i) those related to the production of primary radicals, that rely on enzymatic catalysis; and (ii) other processes (chain transfer, propagation and termination) that are not linked to the enzyme action. As a matter of fact, it has been shown that the propagation kinetics exhibited the same characteristics as previously reported for acrylamide with more classical initiators [12,13].

In this paper, we focus on the enzyme-catalyzed initiation. Starting from results of polymerization experiments, the various reactions involved are analyzed and investigated using UV spectroscopy and ^{1}H NMR experiments, which allow to highlight several processes involving either the enzyme or the other reactants. On the basis of the information obtained, it will be possible to design optimal reaction conditions providing a maximum efficiency of the enzymatic catalysis.

2. Experimental

2.1. Materials

Acrylamide (AAm, Aldrich, +99%, electrophoresis grade), horseradish peroxidase (Sigma, 87 purpurogallin units mg^{-1}, type I), hydrogen peroxide (Prolabo, 30 wt% stabilized aqueous solution), 2,4-pentanedione (Aldrich) and methanol (Prolabo, analytical grade) were used without further purification. The commercial H_2O_2 solution was titrated volumetrically and it was found to have a concentration of 8.1 mol l^{-1} in fairly good agreement with the manufacturer's indications. We checked that the concentration of this solution did not vary significantly between the various experiments.

2.2. Polymerization

A typical procedure for polymerization experiments can be described as follows. To 15 ml of an acrylamide solution (reagent grade water with a resistivity of 18 MΩ cm^{-1}, prepared with Millipore Milli-Q system) introduced into a flask, the required amount of acetylacetone is added. The mixture is stirred by a magnetic stirrer and degassed during 30 min. Then, 0.5 ml of a concentrated HRP solution and the required volume of the H_2O_2 solution are successively added under stirring. Because of the small volumes involved, the H_2O_2 solution is added by means of a microsyringe. After 24 h, the reaction mixture is added dropwise to a large excess of methanol to precipitate polyacrylamide (PAAm). The precipited PAAm is filtered off, washed with methanol and dried under vacuum.

The influence of the pH of the polymerization medium was first considered. Several buffers were used so as to cover a pH range from 3.0 up to 11.5. It is clear that only the reaction media with pH values between 5.4 and 8.0 lead to the production of polymer.

These values define the optimal pH range for HRP

Table 1
Polymerization assays with enzymatic redox systems where one component is initially lacking and added further ($[AAm]_0 = 1.23$ mol l^{-1}; $[Acac]_0 = 17$ mmol l^{-1}; $[HRP]_0 = 1.8$ g l^{-1}; $[H_2O_2] = 11$ mmol l^{-1}; room temperature)

Entry	Initial components	Yield (%)[a]	Added component	Yield (%)[a]	η_{red} (ml g^{-1})[b]
1	HRP, Acac, H_2O_2	87	–	–	337
2	HRP, Acac	0	H_2O_2 (24 h)	87	296
3	Acac, H_2O_2	0	HRP (24 h)	84	283
4	HRP, H_2O_2	0	Acac (24 h)	0	–
5	HRP, H_2O_2, Acac (24 h)	0	H_2O_2 (48 h)	0	–
6	HRP, H_2O_2, Acac (24 h)	0	HRP (48 h)	0	–
7	HRP, H_2O_2, Acac (24 h), H_2O_2 (48 h)	0	HRP (72 h)	87	171
8	HRP, H_2O_2, Acac (24 h), HRP (48 h)	0	H_2O_2 (72 h)	94	131
9	HRP, H_2O_2	0	Acac (0.5 h)	100	302
10	HRP, H_2O_2	0	Acac (1 h)	94	269
11	HRP, H_2O_2	0	Acac (2 h)	90	400
12	HRP, H_2O_2	0	Acac (3 h)	0	–
13	HRP, H_2O_2	0	Acac (5 h)	0	–
14	HRP, H_2O_2	0	Acac (9.5 h)	0	–

[a] The yield corresponds to the weight ratio of dry polymer recovered after 24 h reaction to acrylamide introduced initially. The time at which the component has been introduced is indicated in parentheses. For further additions of the same compounds, the amount introduced is the same as the initial one.
[b] Determined at [PAAm] = 0.17 g dl^{-1} in water at 30°C.

Table 2
Yield and PAAm reduced viscosity for different Acac, HRP and H_2O_2 initial concentrations (the reaction conditions were: $[AAm]_0 = 1.28$ mol l^{-1}; room temperature)

Entry	$[Acac]_0 \times 10^2$ (mol l^{-1})	$[HRP]_0$ (g l^{-1})	$[H_2O_2] \times 10^2$ (mol l^{-1})	α [a]	Yield (%)	η_{red} [b] (ml g^{-1})
1	1.7	1.9	4.2	954	0	–
2	1.7	0.5	1.1	950	0	–
3	1.7	7.6	4.4	250	100	224
4	1.7	1.9	1.1	250	100	284
5	1.7	0.5	0.3	259	100	399
6	1.7	0.35	0.3	370	100	507
7	0.2	1.9	1.2	273	0	–
8	0.2	1.9	0.3	68	65	1639
9	0.2	1.9	0.1	23	0	–

[a] $\alpha = [H_2O_2]_0/[HRP]_0$; the ratio is calculated with molar concentrations for HRP and H_2O_2.
[b] Determined at $[PAAm] = 0.17$ g dl^{-1} in water at 30°C.

activity [18]. The three polymers obtained have similar reduced viscosity ($\eta_{red} = 322$, 333 and 330 ml g^{-1} at pH 5.4 (CH_3CO_2H/CH_3CO_2Na), 5.5 (no buffer) and 8.0 (KH_2PO_4/K_2HPO_4), respectively), which means that no important differences in molecular weight are triggered by pH within the optimal range. The enzyme was not very sensitive to the presence of the buffer since the concentration of phosphate buffer, for instance, can be increased up to 1 mol l^{-1} while maintaining good yields. In what follows, the reactions will be carried out without using buffers (pH of the acrylamide water solution: 5.5).

2.3. Measurements

^1H NMR spectroscopy: The ^1H NMR (200 or 300 MHz) spectra were recorded on Bruker spectrometer at room temperature in D_2O solution using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt as reference. The recording parameters were as follows: relaxation delay 2 s, 16k data points, acquisition time 2 s, and 16 transients.

UV experiments: The reactions were carried out in 1 cm cuvettes and spectra were recorded on a UV–Vis spectrophotometer (Varian Cary 1G) between 600 and 300 nm at different times. For HRP, HRP-I, HRP-II, and (HRP-III, respectively: λ_{max} (nm) = 403, 410 (pH 7), 419 (pH 5.4) and 418 (pH 5.4), and ϵ (cm^{-1} mM^{-1}) = 91, 48 (pH 7), 91 (pH 5.4) and 102 (pH 5.4) [19]. Two absorption maxima (λ'_{max} and λ''_{max}) are very useful to characterize compounds II and III. At pH 7: $\lambda'_{max} = 527$ and $\lambda''_{max} = 554$ nm for HRP-II, and $\lambda'_{max} = 546$ and $\lambda''_{max} = 583$ nm for HRP-III [20].

Viscosimetry: PAAm samples were qualitatively compared by their relative viscosities in water at 30°C at a polymer concentration of 0.17 g dl^{-1}. The value taken for the flow time was the average of eight determinations. The capillary was thermostated using a circulating water bath.

3. Results

3.1. Polymerization experiments

In order to check the role of each component of the ternary redox system, several polymerization experiments were carried out (Table 1). After 24 h at room temperature, only the reaction mixture containing the three components polymerized (entry 1). Furthermore, when HRP or H_2O_2 is added to the "incomplete" redox system (entries 2 and 3), some polymer is obtained within 24 h with a yield comparable to that of the reference experiment (entry 1). On the contrary, when Acac is added to a mixture of HRP and H_2O_2 after 24 h, no polymerization occurs (entry 4). The supplemental addition of H_2O_2 (entry 5) or HRP (entry 6) is not sufficient to observe the polymerization. To obtain polymer from those mixtures, HRP (entry 7) or H_2O_2 (entry 8) must complete the reaction medium. This set of experiments (entries 1–8) is an indication of a possible degradation of HRP by H_2O_2 which cancels out the catalytic activity of the enzyme.

To investigate a little bit more precisely the kinetics of the degradation reaction, we performed several experiments where Acac was added after the introduction of HRP and H_2O_2 with increasing times between the two introductions (entries 9–14). It would appear that the polymerization no longer occurs after 3 h although all the components have been introduced. This means that within about 3 h, the degradation reaction has exhausted either HRP or H_2O_2 or both so that no initiation takes place.

Considering that H_2O_2 is both a substrate and an inhibitor of HRP, it was important to check the influence of the amount of hydrogen peroxide introduced in the reaction medium (Table 2). From the results obtained, we can deduce that the H_2O_2 to HRP initial concentration ratio (α) is a key parameter for the optimization of the HRP catalytic activity.

For $[Acac]_0 = 17$ mmol l^{-1}, no polymerization is observed when α is about 950 whatever the effective

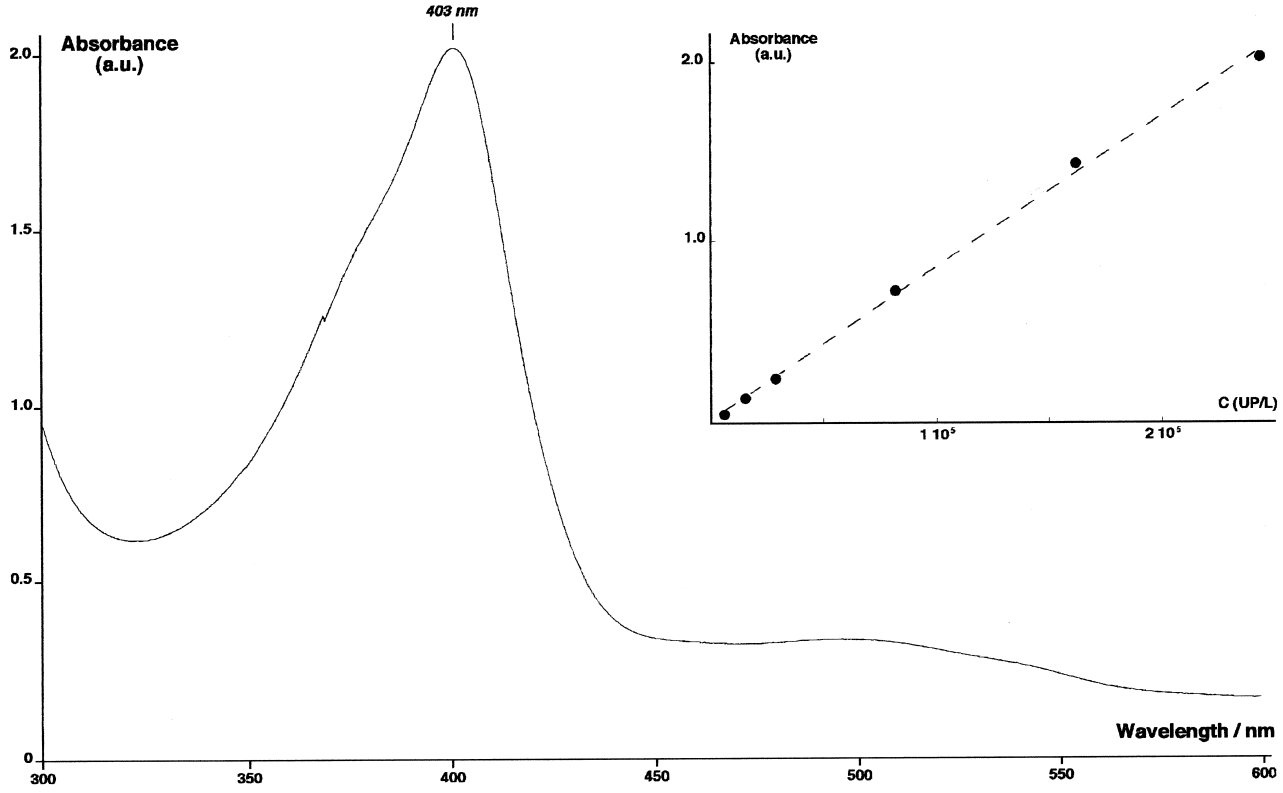


Fig. 1. UV spectrum of an aqueous solution of HRP at a concentration of 2.0 g l^{-1}. The variation of the absorbance at 403 nm with HRP concentration is given in the inset (UP/l means purpurogallin units per liter).

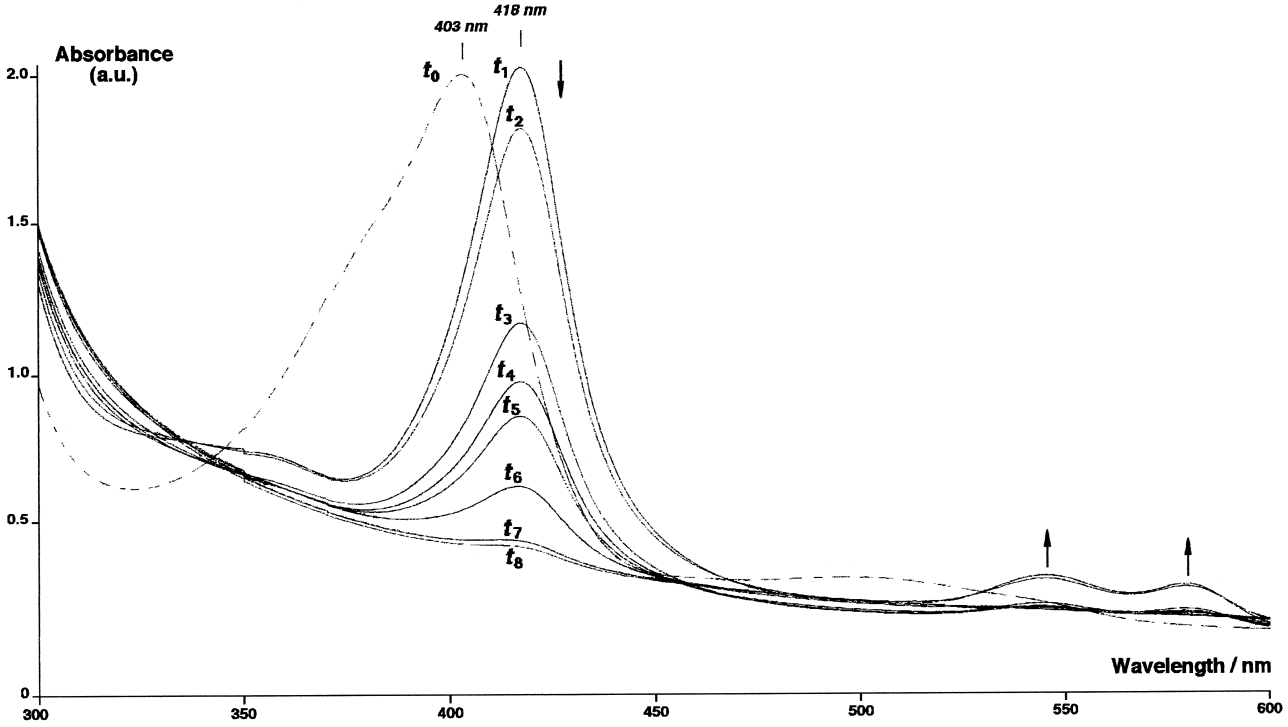


Fig. 2. Evolution of the UV spectrum of an HRP solution after the addition of hydrogen peroxide solution (30 wt%) up to $[H_2O_2] = 0.135$ mol l^{-1} (t_1–t_8 profiles). For convenient representation, several profiles have been deleted. Arrows indicate the way of variation. Profile t_0 (dotted line) corresponds to that presented in Fig. 1 (aqueous solution of HRP).

Table 3
Compounds formed by the addition of H_2O_2 to a HRP solution (2 g l^{-1}) as detected by UV spectroscopy, with or without Acac. Comparison of lactoperoxidase and HRP irreversible inactivation in the presence of H_2O_2 for various concentrations

Entry	This study[a]			Huwiler et al. [21,22][b]	
	α[c]	Compound detected[d]	Enzyme degradation	α'[e]	Enzyme degradation
1	5140	HRP-III	Complete	–	–
2	2570	HRP-III	Complete	2830	Complete
3	1280	HRP-III	Complete	–	–
4	510	HRP-III	Partial	330	Partial
5	210	HRP-III	Partial	170	Partial
6[f]	210	HRP-III	Partial	–	–
7	10	HRP-II	–[g]	–	–
8[f]	10	HRP-II	–[g]	–	–

[a] The enzyme concentration was kept at 52 μM.
[b] The enzyme concentration was kept at 3 μM.
[c] $\alpha = [H_2O_2]_0/[HRP]_0$; the ratio is calculated with molar concentrations for HRP and H_2O_2.
[d] Immediately after the addition of H_2O_2 (on the time scale of the experiment).
[e] $\alpha' = [H_2O_2]_0/[\text{lactoperoxidase}]_0$; the ratio is calculated with molar concentrations for lactoperoxidase and H_2O_2 according to Refs. [21,22].
[f] In the presence of 17 mM Acac.
[g] Not significantly characterized.

concentrations of HRP and H_2O_2 (entries 1 and 2); however, the concentration of HRP can be decreased to 0.35 g l^{-1} (8.1×10^{-6} mol l^{-1} active sites) as long as α is decreased (entries 1–6). The convenient value of α probably depends on the concentration of Acac, since there may be a competition between the degradation processes involving the enzyme and H_2O_2 and the initiating reactions involving the enzyme compounds and Acac. This can be deduced from experiments where the concentrations of Acac and H_2O_2 were both varied. When the Acac concentration is divided by 8 (entries 7–9), no polymerization occurs unless the concentration of H_2O_2 is also decreased. This indicates that the competition between degradation and initiation can be regulated by adjusting the concentration of HRP and Acac to that of H_2O_2. Nevertheless, for Acac and H_2O_2 concentrations around 10^{-3} mol l^{-1}, no initiation takes place since no polymer is recovered (entry 9). At these concentrations, the initiation rate is too low to give rise to significant polymerization. In addition, it appears that the reduced viscosity of the polymer increases when the concentration of enzyme is decreased, irrespective of the concentration of H_2O_2.

At this stage, it appears that degradation reactions occur concurrently to the formation of Acac radicals in the ternary redox system. These reactions involve probably H_2O_2 and are liable to cancel out the polymerization.

3.2. Catalytic cycle followed by UV spectroscopy

The catalytic cycle of HRP involves two intermediate

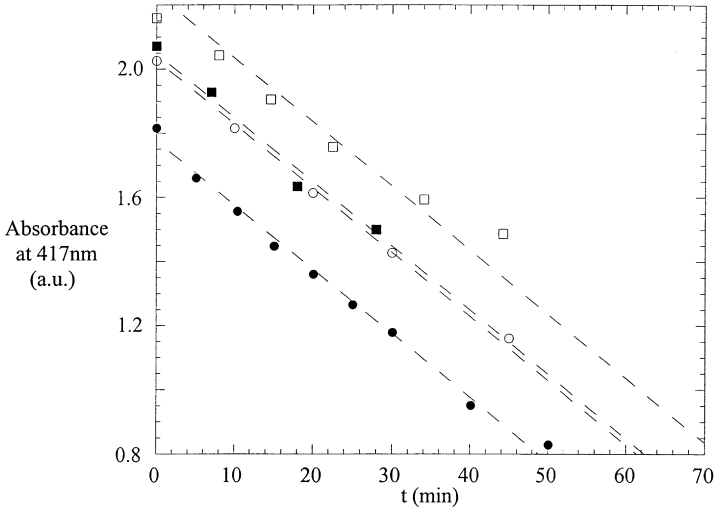


Fig. 3. Initial variation of the absorbance at 418 nm with time for an aqueous solution of HRP after the introduction of hydrogen peroxide solution (30 wt%) up to 0.270 (●), 0.067 (○), 0.027 (■) and 0.011 mol l^{-1} (□).

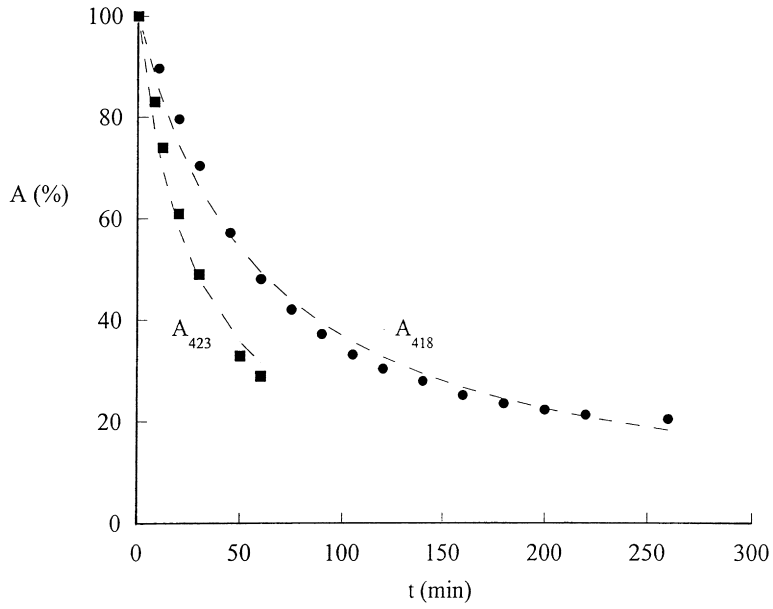


Fig. 4. Decay of the absorbance of the compounds III of HRP (●) and of lactoperoxidase (■) (calculated from the results of Huwiler et al. in the presence of H_2O_2 [21,22]). [HRP] = 52 μmol $^{-1}$ and $[H_2O_2] = 0.135$ mol l^{-1}; $\alpha = 2570$ μmol l^{-1} [lactoperoxidase] and $[H_2O_2] = 8.5$ mmol l^{-1}; $\alpha' = 2830$. The lines result from the fit of the experimental points by assuming a rate of degradation proportional to the square of the concentration of compound III. The absorbances are given as percentages of their initial values and measured at two different wavelengths: 418 and 423 nm for HRP and lactoperoxidase compounds III, respectively.

complexes usually denoted compounds I (HRP-I) and II (HRP-II). Moreover, a third compound may be formed, denoted compound III (HRP-III). The different UV spectra of these compounds allow to follow them by spectrophotometric experiments [19]. A HRP aqueous solution exhibits a maximum absorbance at 403 nm (A_{403}, Fig. 1).

The value of A_{403} depends linearly on the enzyme concentration (see inset of Fig. 1). The A_{403} curve holds for different enzyme lots as long as the concentration is expressed in units of activity l^{-1} instead of g l^{-1}.

The main characteristics of the UV spectra of the various HRP intermediate species are given in the experimental section. With an initial enzyme concentration of 2 g l^{-1}, the addition of H_2O_2 leads immediately (on the time scale of the experiment) to the formation of HRP-III for all the H_2O_2 concentrations higher than 11 mmol l^{-1} (Fig. 2). Moreover, considering the values of the extinction coefficients (experimental section), we can deduce that the conversion of HRP into HRP-III is quantitative. For concentrations as low as 6×10^{-4} mol l^{-1}, only HRP-II was formed after the addition of hydrogen peroxide (Table 3). Compounds II and III of HRP were unambiguously discriminated due to their distinct spectra in the 500–600 nm region [20].

This demonstrates that the different intermediate complexes (active or inactive) are formed in the presence of H_2O_2: HRP → HRP-I → HRP-II → HRP-III. The fact that compound II is formed without the introduction of any hydrogen donor can be explained either by the presence of an endogeneous donor in the enzyme sample or by H_2O_2 itself acting as a donor. This reaction was previously reported for lactoperoxidase [21,22] but has not been mentioned until now in the case of HRP. Nevertheless, it is well known that HRP-III is formed when the enzyme is reacted with a large excess of H_2O_2. For all H_2O_2 concentrations higher than 11 mmol l^{-1}, the absorbance at 418 nm (A_{418}) was found to decrease with time until it completely disappeared. This was an indication of a degradation of HRP in the presence of H_2O_2 and without any hydrogen donor. For all the concentrations tested in that range, and up to 40 min, the decrease of A_{418} is almost linear with a slope value independent of H_2O_2 concentration (Fig. 3). For one concentration ($[H_2O_2]_0 = 0.135$ mol l^{-1}) the decrease of A_{418} was followed up to 260 min (Fig. 4).

The experimental curve can be fitted in a fairly good way by a one-parameter equation resulting from the assumption that the degradation rate of HRP-III is proportional to the square of its concentration. This indicates that the degradation reaction involves either two molecules of HRP-III or one molecule of HRP-III and one molecule of another compound whose concentration is, more or less, proportional to that of HRP-III. For $[H_2O_2]_0 = 27$ and 11 mM (our experiments), HRP-III was reconverted partly into native HRP. The HRP recovery can be roughly estimated by the ratio of the absorbances (50 and 75% for $[H_2O_2]_0 = 27$ and 11 mM, respectively). For $[H_2O_2]_0 = 0.6$ mM, only HRP-II was formed and progressively reconverted to native enzyme. After sufficient time, the HRP recovery is around 100%. In the presence of Acac, the results are the same, at least for the two H_2O_2 concentrations tested (11 and 0.6 mM).

Table 4
^{1}H NMR analysis of HRP/H_2O_2/Acac ternary mixtures. Mole fraction calculated from the integration of the methyl peaks and relative to the total amount acetylacetone introduced. Comparison with calculated values and data from the literature

Entry	$[HRP]_0$ (g l^{-1})	$[H_2O_2]_0$ (mol l^{-1})	$[Acac]_0$ (mol l^{-1})	Acac (%)	Enol (%)	P-IV (%)
1	–	–	0.17	86	14	0
2	–	0.17	0.17	25	5	70
3[a]	–	23.0×10^{-5}	23.0×10^{-5}	30	6	64
4	–	0.51	0.17	5	1	94
5[a]	–	147.0×10^{-5}	49.0×10^{-5}	6	1	93
6	22[b]	0.17	0.17	34	12	54
7	–	–	–	38[c]	8[c]	–
8	22[b]	0.51	0.17	9	4	87
9	–	–	–	11[c]	2[c]	–
10	22	–	0.17	83	17	0
11	22	0.17[b]	0.17	35	11	54
12	22	0.17	0.28[b]	85	15	≈ 0

[a] Composition of Acac/Enol/P-IV mixtures calculated from Milas's results [23].
[b] This component was added 24 h after the other two.
[c] See text for detailed explanations.

Huwiler et al. reported similar experiments with lactoperoxidase [21,22]. They showed that lactoperoxidase undergoes such degradation in the presence of H_2O_2 through the formation of lactoperoxidase compound III. They related the loss of the enzyme (due to degradation) to the initial concentration of H_2O_2 at a constant concentration of lactoperoxidase. Moreover, although their experiments were carried out at concentrations of enzyme and H_2O_2 different from those used here, the results of both studies concerning enzyme degradation can be compared if the ratio α is used instead of the absolute concentrations, and exhibit some similarities (Table 3). The time course of the degradation of lactoperoxidase compound III can be fitted with the same kind of equation (Fig. 4). Nevertheless, the degradation of lactoperoxidase compound III seems to be much faster than that of HRP-III in view of the rate constants found ($k_{\text{lactoperoxidase}} \approx 35k_{\text{HRP}}$).

Concerning lactoperoxidase irreversible degradation (through the formation of compound III), the authors assume that it is not caused by H_2O_2 itself, but rather by a *damaging intermediary reaction product resulting from the catalytic or pseudocatalytic H_2O_2 metabolism* [21].

To account for our results, we should admit that such species has a concentration proportional to that of HRP-III. More experiments would be required to establish the degrading processes involved in the case of HRP. Since, in the presence of Acac, only HRP-II and not HRP-I is detected in the UV experiments, the reactions 3 should kinetically limit the enzymatic cycle (see further Table 5). Even if the rate constants of reactions 2 and 3 are not known for Acac, it has been shown in the case of isobutyraldehyde that the rate constant for the reaction of enol with HRP-II is smaller than for the reaction with HRP-I (the k_2/k_3 ratio is 6.2). This is a general result for hydrogen donor substrates of HRP [20].

The main conclusion of this UV study of the enzymatic system is that a schematic picture of the importance of the hydrogen peroxide to HRP concentration ratio (α) can be drawn as follows: when α is rather high (say higher than 100), HRP-III is formed and starts to irreversibly inactivate. Only a part of the initial HRP content can be recovered depending on the amount of H_2O_2 introduced. This part may be able to initiate polymerization through the enzymatic cycle as described above. For high α values (say 1000) the enzyme is completely degraded. In these situations, no polymerization takes place since the degrading reactions are predominant. When α is low (say around 10), mainly HRP-II is formed and the whole HRP content is involved in the radical polymerization. These results give a more detailed picture of what is happening in the redox system than what was obtained by polymerization experiments. So the value of α is a key parameter in the optimization of the reaction conditions since it is directly linked to the limitation of undesired reactions implying the degradation of the enzyme.

3.3. ^{1}H NMR study of the HRP/H_2O_2/Acac ternary system

It has been established by Milas et al. that in the presence of H_2O_2, Acac can be oxidized into a cyclic peroxide denoted "peroxide IV" (it can be further oxidized into several other peroxides, i.e. peroxides V–VII) [23]. Moreover, the authors claimed that only the keto form of acetylacetone can be oxidized and that the reaction is an equilibrium. This "chemical oxidation" of Acac by H_2O_2 is likely to occur in our redox system and the ^{1}H NMR analysis aimed at evaluating the extent of the competition with the enzymatic process. The ^{1}H NMR spectrum of Acac dissolved in D_2O has been recorded and assigned elsewhere. It may be mentioned here that the methyl resonances of acetylacetone are located at 2.3 and 2.1 ppm for the keto (denoted Acac) and the enol form (denoted *Enol*), respectively; and at 1.6 ppm, if attached to the peroxide ring (P-IV). The proportions of the three components (Acac,

Table 5
Main reactions involved in the enzyme-catalyzed redox system and their rate constants as reported in the literature

No.	Reaction	Reaction rate	Rate constant	Reference
1	HRP + H_2O_2 → HRP-I + H_2O	k_1[HRP][H_2O_2]	$1.8\times10^7 M^{-1} s^{-1}$	[25]
2	HRP-I + enol → HRP-II + Acac$^{\cdot}$	k_2[HRP–I][enol]	–	[25]
3	HRP-II + enol → HRP + Acac$^{\cdot}$ + H_2O	k_3[HRP–II][enol]	–	[25]
4	HRP-I + H_2O_2 → HRP-II + $HO_2^{\cdot}$	–	–	[22][a]
5	HRP-II + H_2O_2 = HRP-III	k_4[HRP–II][H_2O_2]	$46\ M^{-1} s^{-1}$	[25]
		k_{-4}[HRP–III]	$3.0\times10^{-4} s^{-1}$	[25]
6[b]	HRP-III + X → inactive species	k_6[HRP–III]^2	–	[21,22][a], this study[c]
7	Acac = enol	–	–	[23]
8	Acac + H_2O_2 = P-IV	–	–	[23]
9	2Acac$^{\cdot}$ → Acac–Acac	k_9[Acac$^{\cdot}$]^2	–	[25]
10	Acac$^{\cdot}$ + AAm → AAm$^{\cdot}$	k_{10}[Acac$^{\cdot}$][AAm]	–	[25]
11	$HO_2^{\cdot}$ + Acac → H_2O_2 + Acac$^{\cdot}$	–	–	[25]
12	2$HO_2^{\cdot}$ → H_2O_2 + O_2	k_{14}[$HO_2^{\cdot}$]^2	$2.0\times10^7\ M^{-1} s^{-1}$	[25]

[a] The role of proton donor played by H_2O_2 has been evidenced only in the case of lactoperoxidase.
[b] In this equation, X represents an unidentified species which could be either HRP-III itself or another component whose concentration is proportional to that of HRP-III (for details see main text).
[c] The same role as described in footnote (a) has been evidenced in this study.

Enol and P-IV) for various amounts of H_2O_2 added are given in Table 4 as calculated from the integrations of the methyl peaks.

When acetylacetone is dissolved in D_2O, there is about 86% of keto form (entry 1). This value is in agreement with that reported in the literature [15]. The introduction of one equivalent of H_2O_2 (entry 2) leads to the formation of a large amount of P-IV: 70% of the acetylacetone initially introduced is converted into P-IV (this compound exists as a mixture of *cis*- and *trans*-isomers [24]). Nevertheless, this oxidation reaction is an equilibrated process since even in the presence of a three-fold excess of H_2O_2, acetylacetone is not completely converted into P-IV (6% remaining, entry 4). We can notice that these values are coherent with those calculated from the results of Milas et al. obtained from UV experiments with much lower reactant concentrations (entries 3 and 5) [23]. The addition of HRP to the Acac/H_2O_2 mixtures (entries 6 and 8) gives rise to changes in the composition of the mixture. In each case, the mole fraction of P-IV is decreased and the mole fractions of *Enol* and Acac increase. Since the results of entries 6 and 11 are identical, one can conclude that the same "equilibrium" composition is reached starting either from a Acac/*Enol*/P-IV mixture (entry 6) or from a Acac/*Enol* mixture only (entry 11). This means that the oxidation equilibrium leading to the formation of P-IV is established even when it competes with the enzymatic cycle and gives exactly the same composition for the whole mixture. On the contrary, when Acac is added to a HRP/H_2O_2 mixture (entry 12), no P-IV is formed indicating that no H_2O_2 remains in the mixture. These results are consistent with the polymerization experiments presented in Table 1. To try to account for these results, we must consider the keto–enol and Acac oxidation equilibria.

From entries 1 and 10, the equilibrium constant of Acac enolization can be estimated. When HRP is added to the solution, *Enol* is likely to oxidize into Acac$^{\cdot}$ through the enzymatic process, in the presence of H_2O_2. In the absence of the monomer, we assume that all the radicals generated by this process are consumed by combination resulting in the formation of an Acac dimer.

We made two other assumptions concerning the Acac–Acac dimeric species: (i) it is involved in a keto–enol equilibrium with the same equilibrium constant as that of Acac; (ii) the methyl peaks of the Acac–Acac dimer and of its enolic form are located at the same chemical shifts as those related to Acac and *Enol* (respectively). Then, the addition of HRP leads to the consumption of *Enol* with a resulting displacement of the two keto–enolization and oxidation equilibria. These displacements decrease the mole fraction of P-IV in the mixture and apparently increase the ^{1}H NMR signals of Acac and *Enol* since those of the Acac–Acac dimer and of its enolic form now add to them.

We can recalculate the final composition of the mixtures (entries 6 and 8) using the signal of P-IV to estimate the displacement of the oxidation equilibrium with enol consumption (entries 7 and 9). The results are in good agreement with the experimental data within the experimental error. So the assumption that the presence of HRP leads to a consumption of enol and consequently to a displacement of the two equilibria cited above account for the ^{1}H NMR results. From this, the results reported in entry 11 indicate that the enolization and the oxidation equilibria are established before the enzymatic process takes place since the final composition is identical to that reported in entry 6.

4. Discussion and conclusions

4.1. Reactions involved in the HRP/H_2O_2/Acac ternary system

The main reactions involved in the initiating system are

summarized in Table 5 and can be classified into four groups: those involved in the catalytic cycle (reactions 1–4) with Acac (reactions 1–3) or H_2O_2 as proton donor (reaction 4); those implied in the enzyme degradation (forward reaction of equilibrium 5 and reaction 6); those corresponding to the oxidation and keto–enol equilibria of Acac (reactions 7 and 8); and those consuming the radicals produced by the catalytic cycle (reactions 9–12). In our approach all reactions implying oxygen have been neglected since the polymerization medium is always deoxygenated.

Reactions 1–3 describe the enzymatic cycle, which leads to the production of the primary radicals from the acetylacetone ($Acac^{\cdot}$). It has to be noted that the enol form of acetylacetone is implied in the production of $Acac^{\cdot}$. $HO_2^{\cdot}$ can also be generated by this redox system (reaction 4). Since the combination of two $HO_2^{\cdot}$ radicals is very fast, it appears that only $Acac^{\cdot}$ is able to initiate the polymerization in the presence of an acrylic monomer, for instance, and may be the true primary radical of the polymerization (reaction 10). This initiation is thus totally dependent on the enzymatic activity. Nevertheless, the propagation and termination steps are not influenced by enzyme characteristics.

The production of HRP-III (forward reaction of equilibrium 5) is combined with irreversible inactivation of the enzyme (reaction 6). These processes imply a loss of active enzyme for the production of $Acac^{\cdot}$, and thus are unfavorable to the initiation. As a result, the reaction conditions must be adjusted so as to minimize these degradative processes.

Reactions 7 and 8 are the equilibrium of enolization and oxidation of Acac, respectively. The oxidation of Acac by H_2O_2 into peroxide IV is an equilibrated process as previously reported (reaction 8). In addition, it is likely that only the keto form is oxidized to P-IV. The formation of other peroxides from Acac (i.e. peroxide V) has not been considered in our conditions. The equilibrium constants of these processes are important for the initiation because they control the concentration of enol in the solution and hence, the initiation rate.

In order to maximize the concentration of enol in the reaction medium, apart form the keto–enol equilibrium, the β-diketone consumption by the oxidation reaction must be as low as possible. The radicals produced by all these reactions, i.e. $Acac^{\cdot}$ (reactions 2 and 3) and $HO_2^{\cdot}$ (reaction 4) are consumed in several processes depicted by reactions 9–12. The initiation of the polymerization results from reaction 10.

4.2. Optimization of the reaction conditions

The effective concentration of enol is an important parameter since it is the true reactive species for the production of primary radicals. This concentration appears to be related directly to that of Acac but it is also influenced, even to a lesser extent, by the concentration of H_2O_2. Indeed, hydrogen peroxide appears to have three contributions in the initiating system: (i) it is one of the two substrates of HRP (the other being Acac) and leads to the formation of compound I; (ii) it is an inhibitor of HRP since compound III is formed in the presence of an excess of H_2O_2, and leads to an irreversible inactivation of HRP; this can be interpreted as a loss of a part of the HRP initially introduced; (iii) it consumes a part of the Acac initially added by the formation of P-IV.

It is generally accepted that the formation of compound I is not the limiting step of the enzymatic cycle due the high value of k_1 (close to 10^7 l mol^{-1} s^{-1} typically). As a result, the concentration of hydrogen peroxide has to be adjusted by considering the above-mentioned points (ii) and (iii). For a simplified approach of this enzyme-catalyzed redox initiating system, we can consider that the enolization and oxidation equilibria are established within a very short time so that the concentrations of enol, Acac and P-IV are related through the mass-action law. Provided that the equilibrium constants are available, the concentration of enol can be calculated from those of Acac and H_2O_2. The fraction of enzyme that is active for the production of $Acac^{\cdot}$ depends on the competition between reactions 2 and 3 and reactions 4–6.

A more quantitative analysis of this aspect requires the knowledge of the rate constants. Up to now, we can just indicate that low concentrations of H_2O_2 (for instance 7×10^{-4} mol l^{-1}) allow the polymerization to occur without the formation of compound III.

4.3. Conclusions

The main conclusion of this analysis is that the concentrations of the reactants (Acac, *Enol*, H_2O_2 and active HRP) at the beginning of the polymerization are probably very different from those that could be calculated from the Acac, H_2O_2 and HRP concentrations in the feed. This is the result of the implication of all reactants in several reactions either initiating or degrading the redox system. The formation of an inert HRP derivative (compound III) was evidenced by UV experiments and we defined the conditions for limiting these processes while keeping a good yield of polymerization. As for the oxidation of Acac, it was investigated by ^{1}H NMR experiments and it would appear that the concentration range that limits the formation of compound III, was also convenient to have a low concentration of cyclic peroxide resulting from the oxidation of Acac.

A kinetic study of this enzyme-catalyzed initiating system requires the knowledge of the various equilibrium constants as well as the rate constants of the degradation processes. With these requirements, the kinetics equations characteristic of the enzyme cycle could be incorporated into classical equations of free radical polymerization so as to predict the molecular weight of the polymer obtained as a function of the initial concentrations. Such results will be reported in a forthcoming paper [26].

References

[1] Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine Oxford: Clarendon Press, 1989.
[2] Barman TE, editor. Enzyme handbook, vol. 1. New York: Springer, 1985.
[3] Baek HK, Van Waert HE. J Am Chem Soc 1992;114:718.
[4] Saunders BC, Holmes-Siedle AG, Stark BP, editors. Peroxidase London: Buttersworth, 1964.
[5] Kobayashi S. J Polym Sci Part A: Polym Chem 1999;37:3041.
[6] Lalot T, Brigodiot M, Maréchal M. Enzymatic catalysis in organic media for polymer synthesis. In: Aggarval SL, Russo S, editors. Comprehensive polymer science, New York: Elsevier, 1996 (2nd suppl.).
[7] Premachandran RS, Banerjee S, Wu XK, John VT, McPherson GL, Akkara J, Ayyagari M, Kaplan D. Macromolecules 1996;29:6452.
[8] Kobayashi S. Adv Polym Sci 1995;121:1.
[9] Derango AR, Chiang LC, Dowbenko R, Lasch JG. Biotechnol Tech 1992;6:523.
[10] Uyama H, Lohavisavapanich C, Ikeda R, Kobayashi S. Macromolecules 1998;31:554.
[11] Ikeda R, Tanaka H, Uyama C, Kobayashi S. Macromol Rapid Commun 1998;19:423.
[12] Emery O, Lalot T, Brigodiot M, Maréchal E. J Polym Sci Part A: Polym Chem 1997;35:3331.
[13] Lalot T, Brigodiot M, Maréchal E. Polym Int 1999;48:288.
[14] Teixeira D, Lalot T, Brigodiot M, Maréchal E. Macromolecules 1999;32:70.
[15] Toullec J. Keto–enol equilibrium constants. In: Rappoport Z, editor. The chemistry of enols, New York: Wiley, 1990.
[16] Baader WJ, Bohne C, Cilento G, Dunford HB. J Biol Chem 1985;260:10217.
[17] MacDonald ID, Dunford HB. Arch Biochem Biophys 1989;272:185.
[18] Hewson WD, Dunford HB. J Biol Chem 1976;251:6036.
[19] Maehly AC. Methods Enzymol 1955;2:801.
[20] Dunford HB. Haem enzymes. In: Sinnott M, editor. Comprehensive biological catalysis, London: Academic Press, 1998.
[21] Huwiler M, Jenzer H, Kohler H. Eur J Biochem 1986;158:609.
[22] Huwiler M, Bürgi U, Kohler H. Eur J Biochem 1985;147:469.
[23] Milas NA, Mageli OL, Golubovic A, Arndt RW, Ho JCJ. J Org Chem 1963;85:222.
[24] Cocker W, Grayson DH. J Chem Soc Perkin Trans I 1975:1347.
[25] Aguda BD, Larter R. J Am Chem Soc 1990;112:2167.
[26] Durand A, Lalot T, Brigodiot M, Maréchal E. To be published.