Toxicity of fatty acid hydroperoxides towards \textit{Yarrowia lipolytica}:
Implication of their membrane fluidizing action

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Abstract

Linoleic acid hydroperoxide (HPOD), substrate of hydroperoxide lyase, an enzyme of the lipoxygenase pathway, can be transformed into many aromatic compounds, the so-called “green notes”. The presence of linoleic acid hydroperoxide in the culture medium of \textit{Yarrowia lipolytica}, the yeast expressing the cloned hydroperoxide lyase of green bell pepper, undoubtedly exerted an inhibition on the growth and a toxic effect with 90% of yeast cells died after 120 min of exposition in 100 mM HPOD solution. The increase in cell membrane fluidity evaluated by measuring fluorescence generalized polarization with the increasing concentration of HPOD in the medium confirmed the fluidizing action of HPOD on yeast membrane. In addition, we determined by infrared spectroscopy measurement that this compound rapidly diffused into model phospholipids \{1, 2-Dimyristoyl-D54-sn-Glycero-3-Phosphocholine (DMPC-D54)\} bilayer, modifying their general physical state and their phase transition. In the presence of various concentrations of HPOD, the phase transition of DMPC-D54 occurred with an increase of both the corresponding wave number shift and the temperature range but the phase transition temperature was not modified. These results show that the toxic effects of HPOD on the yeast \textit{Yarrowia lipolytica} may be initially linked to a strong interaction of this compound with the cell membrane phospholipids and components.

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1. Introduction

The bioconversion of polyunsaturated fatty acids containing a 1(Z), 4(Z)-pentadiene moiety, into regio-specific hydroperoxides by lipoxygenase and the scission of these hydroperoxides by hydroperoxide lyase to produce volatiles aldehydes are the main steps of the biotechnological process for producing the natural flavours called “green notes”.

Plant hydroperoxide lyases are the major enzymatic source for natural synthesis of these volatile aldehydes, but the plant tissues are not easy to recover and to manipulate [1]. Furthermore, the quantity of recovered enzyme is low and the cost of biocatalyst production very high for the industrial process. The recombinant expression of the biocatalyst is the preferred way to obtain high quantities of stable and efficient enzyme.

Recently, a strong interest in the development of new host for the secretion and production of heterologous proteins has emerged. Among them, the non-conventional yeasts, \textit{Yarrowia lipolytica} is one of the more studied species because it is of interest for biotechnological applications and also due to its ability to secrete high levels of large proteins such as alkaline extracellular protease and RNAse [2,3]. \textit{Y. lipolytica} is able to use fatty acids as sole carbon source [4]. The hydroperoxide lyase from green bell pepper fruit has been cloned and expressed in \textit{Y. lipolytica} and 1200 U of this enzyme per litre of yeast culture medium were recovered after 96 h in shaken flasks [5]. Moreover, 350 mg/L of C6-aldehydes (hexanal and trans-2-hexenal) were produced when hydroperoxides of fatty
acids were added directly in the medium of growing cells by these authors.

However, fatty acid hydroperoxides which are very reactive species can form delocalized lipid radicals or react with other hydroperoxides to form the peroxy radicals [6]. Some studies have demonstrated that cells are sensible to oxidant molecules such as hydroperoxides and free radicals precursors: for example, exogenous octadecadienoic hydroperoxide induced, in a concentration-dependent system, membrane potential derangement [7]. Also, the derived radicals may penetrate into the hydrophobic region and trigger the initiation reaction by abstracting hydrogen from lipids to form new lipid alkyl radicals [8]. On the other hand, one major target of the oxidative species attack is unsaturated lipids, leading to lipid peroxidation causing many pathogenesis and particularly cancer [9], inflammation [10] and also is the primary cause of derangement [7]. Also, the derived radicals may penetrate into the mitochondrial cell [11]. The trihydroxy derivatives of linoleic and linolenic acids have previously been reported to be growth-inhibitory to plant-pathogenic fungi, and a role of the new pathway of linoleic acid oxidation in defense reactions against pathogens is conceivable. [12]. Furthermore, the fatty acid hydroperoxides are decomposed to toxic epoxy acids and unsaturated aldehydes, both species reacting with glutathione. The resulting products seem to induce apoptosis [13].

The aim of this study was to evaluate the toxicity of linoleic acid hydroperoxides on Y. lipolytica by studying their influence on the viability of the cells, the growth and the effect of these hydroperoxides on the yeast membranes. Finally, the behaviour of model phospholipids membrane in the presence of HPOD has been studied.

2. Materials and methods

2.1. Strain and culture conditions

The strain JMY 861 of Y. lipolytica expressing a 6-His-tagged green bell pepper hydroperoxide lyase was grown on YTGA (5 g L⁻¹ yeast extract, 10 g L⁻¹ tryptone, 10 g L⁻¹ glucose, 15 g L⁻¹ agar) medium at 27 °C for 48 h. The biomass was harvested and re-suspended in physiologic water (9 g L⁻¹ NaCl), the suspension was inoculated in 50 mL YTG (5 g L⁻¹ yeast extract, 10 g L⁻¹ tryptone, 10 g L⁻¹ glucose) liquid medium. After 24 h of pre-culture, the biomass was inoculated with an initial O.D. (600 nm) of 0.25 in 50 mL YTG medium (2.5×10⁶ cells mL⁻¹). The cells were grown in 100 mL baffled Erlenmeyer flasks, agitated on a rotary shaker at 140 rpm and 27 °C for 19 h. The biomass was then harvested for processing.

2.2. Linoleic acid hydroperoxide preparation

Hydroperoxides were prepared by oxidation of linoleic acid (Sigma Chemical Co., St. Louis, MO) as previously described [14]. 500 mg of linoleic acid were mixed with 15 mg soybean lipooxygenase (Sigma, Chemical Co, St. Louis, MO; 45 000 U mg⁻¹ solid) in borate buffer 0.1 M, pH 9.6. After 1 h at 25 °C, 300 rpm and 30 mL O₂ min⁻¹, the enzyme was inactivated by the addition of H₂SO₄ until pH 4. Linoleic acid hydroperoxides were extracted with diethyl ether (Prolabo, France) for three times. The organic phase was dried with MgSO₄ (Sigma Chemical Co., St. Louis, MO) and evaporated under vacuum. The hydroperoxides were dissolved in absolute ethanol and stored at −20 °C. The conversion of fatty acids to fatty acids hydroperoxides was checked by measuring the absorbance at 234 nm.

2.3. Measurement of yeast viability

Cell viability was estimated in triplicate by the methylene blue staining method and the CFU method. For the first method, after hydroperoxides treatment at different concentrations (25 mM, 50 mM, 100 mM) cells were stained with methylene blue solution carefully prepared and filtered (0.25 g L⁻¹ methylene blue, 9 g L⁻¹ NaCl, 0.42 g L⁻¹ KCl, 0.32 g L⁻¹ CaCl₂, 0.2 g L⁻¹ NaHCO₃ and 10 g L⁻¹ glucose). The quantity of living and death cells was evaluated by microscopy. For the second method, HPOD treated cells were serially diluted and the appropriate dilutions were plated onto three plates. CFU were counted after an incubation of 24 h at 27 °C. The initial cell suspension was used as control.

2.4. Fluorescence generalized polarization measurements

Membrane fluidity was assessed by measuring fluorescence generalized polarization of 2-dimethylamino-6-lauroyl-naphthalene (Laurdan) (Sigma, St. Quentin Fallavier, France). The cells were washed three times (6000 g, 5 min) with 0.05 M phosphate buffer solution pH 6.7 and then with physiologic water (9 g L⁻¹ NaCl). HPOD were added to 2 mL of cell suspension (8×10⁶ cells mL⁻¹) with various concentrations (from 0 to 25 mM) and stirred for 20 min, at 27 °C. Yeast cells were harvested by centrifugation (10,000 g, 5 min) and then washed two times with phosphate buffer pH 6.7 to completely remove HPOD because this compound could react with Laurdan molecule and perturb the fluorescence signal of yeast cells. The remaining HPOD in the samples after centrifugation was determined by measuring the fluorescence of supernatant marked Laurdan. The signal intensity was always less than 5% of the total fluorescence measured in the presence of yeast labelled cells (data not shown). Cells were then labelled with 4.5 μM Laurdan in cuvette according to the method of Palleschi and Silvestroni [15] at 27 °C for 1 h incubation. In each cuvette, sample volume was 3 mL. The measurements were done with a spectrofluorometer (Hitachi Instrument, F4500, Japan) equipped with a stirred and thermostated (27 °C) cuvette holder, and connected to an acquisition and processing system (Hitachi).

Laurdan fluorescence emission spectra were recorded in the range from 400 nm to 550 nm, using both 350 nm and 390 nm excitation wavelengths, whereas the fluorescence excitation spectra were obtained in the range from 320 nm to 420 nm, using both 440 nm and 490 nm emission wavelength. Blank spectra were obtained with unlabeled cells and were subtracted from the spectra of labelled cells. These blank responses were always <5% of sample responses in each wavelength range considered. From the spectroscopic data, Laurdan excitation generalized polarization (GPeX) spectra were constructed by calculating the GP value for each wavelength by the following formula:

\[
GPeX = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}
\]

where \(I_{440}, I_{490}\) are the intensities measured, at each excitation wavelength (from 320 nm to 420 nm), on the fluorescence excitation spectra obtained by fixed emission wavelength of 440 nm and 490 nm, respectively. The choice of 390 nm, 350 nm, 440 nm, and 490 nm for GP calculations was made according to Machado et al. [16]. The membrane fluidity was evaluated as the GPeX value obtained at 350 nm.

2.5. ATR-FTIR studies

Experiments were performed using a Fourier Transform Infrared spectrophotometer IFS Vector 22 (Bruker, Germany), equipped with a ZnSe ATR crystal and a diaisys cell, and linked to a computer with OPUS software (Bruker), permitting spectra acquisition and data treatment. The dialysis cell (Bio-ATIR: Bruker, Germany) allows liquid circulation on the phospholipids film to be studied via a dialysis membrane. This system permits the user to hydrate sample in a non-perturbing manner and to maintain close contact between the crystal and the film being studied. The model phospholipids in this study, dimyristoyl-1,2-phosphatidylcholine (DMPC) (Sigma) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC-D54) (Avanti Polar Lipids, Alabaster, USA) were deposited (150 μl from a 20 g L⁻¹ of chloroform solution) on the IR-transparent ZnSe window at 35 °C for 30 min to form a dry phospholipids film. The window was covered with a water-tight cell, enabling
the introduction of a solution in order to hydrate the lipid film with 2 ml of deionized water or HPOD solution. The hydration of phospholipids needs to be performed at a temperature higher than the phase transition temperature, to ensure a fluid conformation facilitating the entry of water molecules. Furthermore, the hydration was controlled by following the asymmetric stretching vibration of the PO2\(^{-}\) groups as a wave number of about 1220–1230 cm\(^{-1}\) is characteristic of hydrated heat groups [17]. Phospholipids were first cooled to 12 °C and then heated to 35 °C (0.5 °C min\(^{-1}\)). Temperature was controlled using a thermocouple inserted in the cell. During this heating step, ten scans were taken every 1 °C at a resolution of 2 cm\(^{-1}\) and an average spectrum was produced.

3. Results and discussion

3.1. Influence of HPOD on the growth and the viability of \textit{Y. lipolytica}

The growth of \textit{Y. lipolytica} was monitored in a medium containing glucose as carbon source and the cells were in the stationary state after 22 h (0 mM HPOD). Yeast cells growth rate was significantly reduced in the presence of HPOD and the stationary phase was reached after 26 h and 30 h with 25 mM and 50 mM HPOD, respectively. The growth was very weak with 100 mM HPOD (Fig. 1). These results indicated that HPOD presented an inhibition on the yeast growth.

Concerning the influence of HPOD on the yeast viability, after 19 h of culture in the YTG medium, the yeast cells were treated with different concentrations of HPOD, then coloured by using methylene blue: only the living cells can decolorize this reagent. To determine the kinetic of the lost of \textit{Y. lipolytica} cell viability after exposure to different HPOD solutions, samples were taken from the culture at 30-min intervals over 150 min (Fig. 2). The results showed that in the presence of 25 mM HPOD, there was an initial linear loss of cell viability according to the times of HPOD exposition until 150 min. Concentrations above 25 mM caused also a rapid linear loss of cell viability until 120 min of exposed time, then the decrease was reduced. This may have resulted from the presence of two populations with different sensitivities to HPOD in the culture [18]. Furthermore, after a treatment with 25 mM HPOD for 120 min, the cell viability dropped to 63% which corresponded to the viability of 30-min exposed cells in 100 mM HPOD solution. So, a long exposition (120 min) of low concentration (25 mM) of HPOD was able to cause the same effect into yeast cells compared to the action of higher concentration (100 mM) of HPOD during shorter exposition time (30 min). This may be explained by the progressive incorporation of polyunsaturated fatty acids from the medium into the yeast membrane [19,20]. Moreover, the results of cell viability obtained by CFU method have shown the same influence of HPOD on the yeast \textit{Y. lipolytica}: after a treatment with 100 mM HPOD for 150 min, the cell viability dropped to 10.8% which corresponded to 9.7% of living cells obtained by methylene blue method or 69% of living cells after 90-min contact with 25 mM HPOD compared with 73.5% by methylene blue method (data not shown).

According to the literature, linoleic acid hydroperoxides presented also a toxic effect toward \textit{Saccharomyces cerevisiae} but at very low concentration [18]. The majority of these cells have died after a 40-min contact with 0.2 mM HPOD, while in our study, after 60-min contact with 50 mM HPOD, 64% of \textit{Y. lipolytica} cells were still alive. This phenomenon can be explained by the capacity of this yeast which express the cloned hydroperoxide lyase of green bell pepper [5] to metabolize HPOD into aroma compounds. Furthermore, the action of other peroxides like \textit{H2O2} on the cell viability of \textit{S. cerevisiae} was also studied [18]. Their results showed that the same concentration of \textit{H2O2} (0.2 mM) did not cause any toxic effect for this yeast. The HPOD lethal dose toward \textit{S. cerevisiae} (0.2 mM) was much lower than \textit{H2O2} (6 mM) [21] or other peroxides like the tert-butyl-hydroperoxide (15 mM). This phenomenon can be explained by the action mechanism of these compounds with respect to the yeasts. \textit{H2O2} is a polar compound which cannot easily diffuse into the lipidic bilayers and the tert-butyl group of hydroperoxides can prevent their insertion into the membranes due to its steric hindrance, reducing in both cases their toxic effects. Concerning HPOD, it must exist a combined action of C18 acyl hydrocarbon chain and peroxide polar group. Indeed, their structure strongly resembles the one of linoleic acid, so that they could be...
incorporated into cell membrane more easily than H$_2$O$_2$ and the peroxide group could then influence the cell metabolism. Consequently, it was interesting to determine the action mechanism of HPOD on the yeast cells. In this objective, we studied the influence of HPOD on the cell membrane which is the first barrier to toxic compounds from the culture medium.

3.2. Influence of HPOD on membrane fluidity

Fluorescence generalized polarization (GP) of Laurdan is related to membrane state and fluidity: decreasing values of GP indicate an increasing fluidity [22]. Increased yeast membrane fluidity with increased medium temperature is measured by different spectrofluorimetry methods involving fluorescent probes [23–25]. In our study, GP was evaluated at temperature from 25 °C to 40 °C and a linear relationship between these two parameters was obtained: GP was found to be around 0.37 at 25 °C and 0.25 at 40 °C (Fig. 3a).

The HPOD presented also a strong effect on fluorescence generalized polarization (Fig. 3b): the GP value declined almost linearly with increasing HPOD concentration from 0.38 (without HPOD) to 0.22 (HPOD 25 mM). By comparison with the effect of temperature on membrane fluidity, there was the similar decrease of GP: with 5 mM HPOD, the GP value was 0.32 corresponding to GP value at 30 °C and it dropped to 0.22 in the presence of 25 mM HPOD as shown for 40 °C. Consequently, it can be concluded that HPOD increased membrane fluidity in a concentration-dependent manner.

The effect of decanoic acid on membrane of $S$. cerevisiae was previously described. The presence of decanoic acid caused an increase of membrane fluidity. Apparently, HPOD action appeared to be exerted at the membrane lipids level, making its fluidizing action comparable to the action of decanoic acid. Moreover, analysis of lipid composition of yeast cell membrane indicated a modification in the distribution of fatty acids and sterols of cells in the medium containing 35 μM of decanoic acid [26]. So, in order to understand the action mechanism of HPOD, the same experiment with linoleic acid instead of HPOD was carried out and we noticed that linoleic acid presented almost the same effect of membrane fluidization as HPOD (Fig. 3c). At 5 mM, the GP value was 0.30 and it reached 0.18 in the presence of 25 mM. The GP values of yeast cell with linoleic acid treatment were even lighter than with HPOD treatment. Consequently, we could suggest that the hydrocarbon chains of HPOD which were able to incorporate into cell membrane should have a fluidizing role in membrane phospholipids.

On the other hand, we noticed that after 20-min contact with 25 mM HPOD, the cells expressing a very fluid state of their membrane were still alive (more than 94% of living cells after 30 min), then the viability decreased according to the contact time (51.9% after 150 min) (Fig. 1). The same experiment measuring GP of cell membrane after a longer HPOD exposition was carried out to examine the influence of contact time with this compound on the membrane fluidity. The result showed that the GP value did not change after 30 min of contact (data not shown). This fact suggested that the cell membrane state did not change after reaching a threshold and yeast cells were able to withstand an important increase of membrane fluidity which had no effect on the viability of this yeast in a limited time. Apparently, according to the contact time, the HPOD undoubtedly induced the increasing cell mortality. In that way, we could suggest that due to the membrane fluidity change caused by HPOD, the yeast lose progressively their metabolic activity.

3.3. Interaction of HPOD with phospholipids bilayers

Pure phospholipids were studied by the mean of ATR-FTIR spectroscopy in order to understand the behaviour of the
of DMPC-D54 bilayers brought into contact with pure water (100 mM HPOD). The CH2 groups existing in the HPOD molecules may diffuse into the phospholipid bilayers then perturbing the C–H signal of DMPC detected by FTIR. For these reasons, we also use the deuterated phospholipids DMPC-D54 (1, 2-Dimyristoyl-D54-sn-Glycero-3-Phosphocholine) for FTIR spectroscopy to completely remove the contribution of the CH2 groups of HPOD from the signal.

In a first study, the interaction of HPOD with model phospholipid system according to contact time was evaluated. From the ATIR ZnSe crystal dimensions and from the number of DMPC-D54 molecules (150 μL DMPC-D54 of a solution of 20 g L⁻¹), the lipid film could be assimilated to a multibilayer composed of approximately two thousands superposed bilayers [27]. We reported the symmetric stretching signals values DMPC-D54 against time at 35 °C, with liquid-crystalline lipids which corresponds to the physical state of membrane phospholipids in physiological conditions in yeast and more generally in living systems, from the moment when HPOD solution was brought into contact with phospholipid bilayers (Fig. 4). Therefore, the curves represented the diffusion of HPOD within this structure. As HPOD was added to the model phospholipids, the wave number shifted to higher values indicating an increasing global disorder degree within bilayers. There was a relatively rapid insertion phase and then a slow and longer phase during which the signal values slightly reached a maximal value (2092.7 cm⁻¹): this way correspond to an equilibrium phase. When pure water was introduced into DMPC-D54 bilayers, there was no shift in wave numbers of stretching signal; these remained equal to the values obtained before the introduction of the HPOD solution. Thus, the detected shifts corresponded only to the introduction of HPOD within phospholipid bilayers. Apparently, the evolution of signal values indicated that the molecules of HPOD diffused progressively into each bilayer and perturbed the conformation of hydrocarbon chain of DMPC-D54. This result was in correlation with the toxic effect of HPOD showed previously on Y. lipolytica cells.

In a second study, the effect of HPOD incorporation in DMPC-D54 bilayers was determined by studying their phase transition after diffusion of a HPOD solution. The typical phase transition curves obtained of DMPC-D54 are illustrated in Fig. 5, which was evaluated with various concentrations of HPOD. The results indicated that, in water, there was a phase transition of DMPC-D54 at about 20 °C, with a shift in wave number from 2194.3 cm⁻¹ (νasCD2) at 18 °C to 2195.5 cm⁻¹ at 22 °C. Below 18 °C and above 22 °C, the wave number increased slightly with temperature. In the presence of 20 mM HPOD, transition of DMPC-D54 took place between 15 °C and 24 °C, between 14 °C and 26 °C at 50 mM HPOD. At 100 mM HPOD, it occurred between 12 °C and 27 °C. Thus, the range of temperature in which phase transition occurred increased with increasing HPOD concentration in the medium. Moreover, the wave number corresponding to the state of phospholipids changed with the HPOD concentration. At 20 mM HPOD, the signal values of DMPC-D54 were higher than in the water, but less marked in the liquid-crystalline state. Concentrations above 20 mM caused a global increase of signal values of C–H stretching bands in a HPOD concentration-dependent manner. To facilitate comparisons between samples, the whole results of asymmetric stretching band are synthesized in Table 1 calculating the wave number limits of phase transition and the phase transition temperature. Apparently, the diffusion of HPOD into model membranes led to an important prolongation in the phase transition. At 20 mM, HPOD induced an increase of wave number limits corresponding to phase transition of DMPC-D54 bilayers from 2194.5 cm⁻¹ to 2195.6 cm⁻¹ while it was from 2194.3 cm⁻¹ to 2195.5 cm⁻¹ in the water. At 50 mM, the increase of wave number limit in the liquid-crystalline state was more marked. There was a phase transition with a shift in wave number from 2194.6 cm⁻¹ to 2196 cm⁻¹. In the presence of 100 mM HPOD, the phase transition of DMPC-D54 occurred from 2194.6 cm⁻¹ to 2196.1 cm⁻¹. So, their wave number limits were much higher both in the gel and liquid-crystalline state with increasing HPOD concentration. Moreover, the shift
of wave number corresponding to the phase transition increase with increasing HPOD concentrations: it was 1.4 cm\(^{-1}\) at 50 mM, 1.5 cm\(^{-1}\) at 100 mM but it did not increase at 20 mM. Studying the phase transition temperature of DMPC-D54 (Tm) with various concentrations of HPOD, there was a decrease of temperature limit of transition in the gel state and an increase of this value in the liquid-crystalline. Therefore, with increasing HPOD concentration, the temperature range corresponding to phase transition of DMPC-D54 was higher: it was 9 °C at 20 mM, 12 °C at 50 mM and 15 °C at 100 mM HPOD while in the water it was 4 °C. However, the phase transition temperature, which is the average of temperature limit values, indicated that HPOD did not induce any significant change of this temperature. It was always around 19.5 °C and 20 °C.

In conclusion, the HPOD diffusion into DMPC-D54 bilayer strongly increased the global disorder within the lipid chains and modified the phase transition profile. These results were correlated with the fluidizing effect of HPOD to yeast membrane estimated by Laurdan generalized polarization measurement. The effects of HPOD on fluorescence generalized polarization measurement and on vibration frequencies of the pure phospholipids acyl-chains were in both cases comparable to the effects of temperature, expressing a strong disordering action.

4. Conclusion

These results enabled us to understand the influence of HPOD on Y. lipolytica viability and especially to understand its effects on membranes.

We have shown a strong action of HPOD, inhibiting the growth of the yeast and we have also shown that the viability of Yarrowia in presence of these compounds was strongly decreased with increasing HPOD concentrations.

HPOD may incorporate into membrane phospholipid bilayers leading to a progressive increase in degree of disorder. The resulting global fluidity within cell membranes should modify their function properties (e.g. permeability) and then create a metabolic disorder explaining the relative slow death of the cells. These results may be explained by a strong interaction between the hydrophobic carbon chains of HPOD with that of the acyl chains of phospholipids.

The fluidizing properties of HPOD can be useful to obtain controlled membrane physical state modification in microorganisms: for example, the non-toxic concentration of HPOD (25 mM) can be useful to modify cell membrane fluidity. This fact suggests a potential use of HPOD to prevent membrane damage that can be induced by some environmental stresses which lead to sharp decrease in membrane fluidity [28]. On the other hand, yeast cells resistance to high HPOD concentration could be theoretically improved by decreasing membrane fluidity. These fluidizing effects could be counterbalanced by an appropriate rigidifying perturbation.

The membrane fluidizing action of linoleic acid hydroperoxides demonstrated in this study could constitute a natural function for these compounds.

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