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Predicted secondary structure of hydroperoxide lyase from green bell pepper cloned in the yeast *Yarrowia lipolytica*

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ABSTRACT

Fatty acid hydroperoxide lyase (HPL) is a member of the cytochrome P450 family acting on fatty acid hydroperoxides in many organisms. The active green bell pepper HPL, cloned and expressed in the yeast *Yarrowia lipolytica*, was purified by immobilized metal-ion affinity chromatography (IMAC) in the presence of 2% of Triton X-100R. The secondary structure prediction by bioinformatics servers of HPL was realized by ANTHEPROT software, using the GOR, DPM and Predator methods. The theoretical results which are average values obtained from three different calculation methods showed 33% α-helix, 18% β-sheet, 7% turn and 42% coil. On the other hand, the secondary structure approach of the purified active HPL (specific activity of 2.94 U/mg protein) was realized by differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy, and showed 13% α-helix, 29% β-sheet, 5% turn and 53% random coil.

1. Introduction

Hydroperoxide lyase (HPL) is an enzyme widely found in plants and vegetables. HPL is involved in the biosynthesis of volatile aldehydes and alcohols and it catalyzes the breakdown of fatty acid hydroperoxides into ω-oxo-acids and aldehydes (C6 or C9), which are used industrially to reconstitute the "fresh green odor" of fruits and vegetables lost during processing. HPL is a key enzyme for the biosynthesis and the production of green notes molecules, such hexanal and trans-2-hexenal and plant HPL is the major enzymatic source for the synthesis of these natural volatile aldehydes; however, it is not easy to recover and to handle the enzyme from plant tissues [1]. Moreover, the amount of recovered enzyme is low and the cost of biocatalyst production is high for an industrial process. Hence, the recombinant expression of the biocatalyst is the appropriate way to obtain high quantities of stable and efficient enzyme [2].

Although the genes of HPL from many species of vegetables and fruits, including bell pepper fruit [3], alfalfa [4], *Arabidopsis thaliana* [5], cucumber [6], tomato [7] and guava fruit [8], have been cloned, large quantities of these tissues were used in order to characterize the enzyme. Matsui et al. [3] expressed HPL of bell pepper fruit in *Escherichia coli*; these authors demonstrated that this enzyme is a hemoprotein, member of the cytochrome P450 family. In green bell pepper fruits, HPL is a membrane-bound protein of about 170 kDa [9].

A strong interest in the development of new hosts for the secretion and production of heterologous proteins has emerged. Among them, the non-conventional yeast *Yarrowia lipolytica* is one of the more studied species because of its use in biotechnological applications as well as its ability to produce high levels of large proteins, such as alkaline extracellular protease and RNAse [10,11]. In a previous work [12], the HPL from green bell pepper fruit was purified, with a maximum of 7500 mg aldehydes per g protein has been produced by the purified enzyme; in addition, 392 and 88 mg aldehydes were obtained with use of the chloroplast fraction and the crude enzymatic extract, respectively. Bourel et al. [13] reported the expression of HPL from green bell pepper in the yeast *Y. lipolytica*, where the growing cells showed the aldehydes production.

Regarding the amount of available concentration, after expression and purification, the size of the protein, and the membrane localization, HPL structural studies are challenging. Most of the classical biophysical techniques, including NMR, FTIR, X-ray and fluorescence spectroscopy which are usually used to provide protein structural information, cannot be applied. Nevertheless, the structural characterization of such enzyme can be considered as critical steps for a much better understanding and control of HPL activity. In a previous work, we have used circular dichroism to explain the differences of HPL activity found in different media [14]. Concerning the HPL structure, as the authors are aware, the literature [15] indicated limited information, where the CD spectra of HPL from alfalfa, cloned in the bacteria *E. coli*, showed 75% of

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α-helix, with no obvious structural differences in the presence or absence of 0.2% Triton X-100, used to mimic a membrane environment. Moreover, these authors [15] suggested that the HPL activity could be mainly regulated by the organization of the membrane around the enzyme. On the basis of the little information available in literature regarding the structure of HPL, the present study could be the first step for providing better characterization of HPL.

The aim of the present study was to use bioinformatic servers to predict secondary structure of the HPL and to confirm the predicted structure by experimental structural studies involving circular dichroism. In addition, the differential scanning calorimetry (DSC) of the purified HPL, expressed for the first time in the yeast *Y. lipolytica*, was used to monitor whether the enzyme was structurally folded. Moreover, the purified HPL secondary structure was calculated by circular dichroism (CD) spectroscopy, where the CD spectroscopy is considered as an effective tool for monitoring the changes in the secondary and tertiary structures in protein binding studies [16,17].

2. Materials and methods

2.1. Culture of yeast

The strain JMY 861 of *Y. lipolytica*, expressing a 6-His-tagged (N-terminal) green bell pepper HPL, was grown on YTGA (5 g/L yeast extract, 10 g/L tryptone, 10 g/L glucose, 15 g/L agar) medium at 27 °C for 48 h. The biomass was harvested and inoculated in 200 mL YTG (5 g/L yeast extract, 10 g/L tryptone, 10 g/L glucose) liquid medium for 24 h. The volume of the suspension was determined in order to have an initial OD_{600} of 0.2. After 24 h of pre-culture, the biomass was inoculated with an initial OD_{600} of 4 (10^7 cells/mL) in 100 mL YTO medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L olive oil, 5.3 g/L NH_4Cl). The cells were grown in 500 mL baffled Erlenmeyer flasks, agitated on a rotary shaker at 140 rpm and 27 °C. The biomass was then harvested and used during this study.

2.2. Extraction and purification of HPL

The fresh biomass *Y. lipolytica* was harvested and washed three times with 100 mM Tris–HCl buffer (pH 8.0). The cells were loaded in the Tris-HCl buffer, containing 2% Triton X-100R (Sigma Chemical Co., St. Louis, MO), 300 mM NaCl, 2 mM imidazole and complete EDTA-free protease Inhibitor Cocktail Tablets (1 tablet per 50 mL of the Tris–HCl buffer; Roche Diagnostics, Penzberg, Germany) at a ratio of 1 g per 6 mL of buffer. The suspension was loaded in a one-shot cell disrupter (Z plus series "One shot") at 1.6×10^8 Pa and centrifuged three times for 20 min at 10,000 × *g*. The resulted supernatant was considered to be the crude enzymatic extract. Immobilized Metal Affinity Chromatography (IMAC) was used to purify HPL. Three milliliters of resin Ni-NTA Agarose (QIAGEN, Courtaboeuf, France) was preliminary equilibrated on a column (1 cm × 1 cm) with 15 mL of buffer A (100 mM Tris–HCl pH 8.0, containing 0.2% Triton X-100R, 300 mM NaCl and 2 mM imidazole), with a flow rate of 0.5 mL/min. Thirty milliliters of crude enzymatic extract were loaded onto the equilibrated resin at a flow rate of 0.3 mL/min and the resin was then washed with 15 mL of buffer A and 15 mL of buffer B (50 mM sodium phosphate, 0.2% Triton X-100R, 300 mM NaCl and 15 mM imidazole) at pH 6.0 at a flow rate of 0.5 mL/min. The His-tagged protein was eluted with 30 mL buffer B, pH 5.0, containing 250 mM imidazole, and dialyzed during 48 h for the removal of imidazole and Triton X-100R. The dialyzed enzymatic extract was concentrated 60-fold, using Microcon centrifugal filter devise (Millipore, France). All steps were carried out at 4 °C. The protein concentrations were determined, using a calculated A_{280} coefficient based on the number of Trp, Tyr and Phe residues, where 5, 16 and 39, were deduced, respectively, from the HPL gene sequence published by Matsui et al. [3].

2.3. HPL enzymatic assay

HPL activity was determined using potassium phosphate buffer solution (100 mM, pH 5.5), containing 25 mM of 13-hydroperoxide of linoleic acid (13-HPOD) as substrate, prepared in absolute ethanol. The enzymatic reaction was initiated by the addition of 4 μL of the purified enzymatic protein (0.7 mg protein/mL). The decrease in A_{234}, due to the cleavage of the substrate, was followed spectrophotometrically for 1 min. The activity was calculated from the initial slope of the resulting absorbance curve. One unit of HPL activity was defined as the amount of enzyme that converted 1 μmol of HOPD substrate within one min.

2.4. Differential scanning calorimetry of HPL

The thermal stability of purified samples was examined by differential scanning calorimetry (DSC) in a Setaram DSC3 microcalorimeter. The buffer used as inert reference was the potassium phosphate (100 mM, pH 5.5). DSC was performed with 10 mg/mL of purified enzyme. The protein samples were scanned between 30 and 110 °C at a rate of 1 °C/min.

2.5. Secondary structure prediction

Secondary structure prediction was carried out using the ANTHEPROT software (ANalyze THE PROTeins), which is an efficient predictive method that can be of help in the modeling of the proteins structures. This software is a package to make protein sequence analyses, such as the alignment, the secondary structure prediction, the sites and function detection, the physico-chemical profiles, the homology search and the 3D display of protein structures [18]. Starting from the amino acid sequence, published by Matsui et al. [3], the server (http://antheprot-pbil.ibcp.fr/) was used for the predictions.

2.6. Circular dichroism (CD)

CD spectra were recorded on a Jobin-Yvon spectropolarimeter (Longjumeau, France), using 5 μM of the purified HPL. The CD spectra were analyzed, with the DICROPROT V2.5 application package [19], to estimate secondary structure composition. Protein samples were suspended in potassium phosphate buffer 100 mM (pH 5.5) and scanned at room temperature in quartz Suprasil cells with a path length of 1 mm; the CD spectrum of this buffer was subtracted from the CD spectrum of the enzyme. The spectra were presented as circular dichroism per residue, $\Delta\varepsilon$ ($M^{-1}\,cm^{-1}$), in function of wavelength (λ_{nm}) recorded in the range from 180 to 260 nm. The α-helix content in the polypeptide was estimated from the dichroic increment at 222 nm, by the relation $P_\alpha = -[\Delta\varepsilon_{222} \times 10]$, where P_α is the percentage of the α-helix and $\Delta\varepsilon_{222}$ is the circular dichroism per residue at 222 nm [20].

3. Results and discussion

3.1. HPL purification and activity

The HPL crude enzymatic extract, expressed by *Y. lipolytica*, was solubilized with 2% Triton X-100R before its purification by immobilized metal affinity chromatography (IMAC). Twenty-two milligrams of the purified enzyme were recovered and characterized by UV–vis absorption and SDS-PAGE analysis. The purified HPL showed only one major band on SDS-PAGE and a specific activity of 2.94 U/mg protein (Table 1) corresponding to previous result

Table 1
Protein concentration and HPL activity obtained after purification by IMAC.

sample	Protein concentration (mg/mL)	Total activity (U/mL)	Specific activity (U/mg of protein)
Cellular extract	9.7	1.08	0.15
Purified protein	0.45	1.47	2.94

500 μL of resin and 2500–3000 μL of cellular extract.

obtained in our group [21]. In order to determine whether the purified protein obtained was structurally formed, the active enzyme fractions were used for the biophysics measurements, including DSC and CD.

3.2. Secondary structure prediction

In order to investigate the enzyme secondary structure of the purified bell pepper HPL, cloned in the yeast *Y. lipolytica*, two ways were followed; the first was by bioinformatics servers from the amino acids sequence, whereas the second was by biophysic measurements, including DSC and CD spectroscopy from the purified bell pepper HPL, cloned in the yeast *Y. lipolytica*.

The molecular weight of the HPL from green bell pepper, which contain 480 amino acids, was calculated by ANTHEPROT Software to be 54 kDa. Indeed, in a previous study, a molecular weight of 57 kDa for the purified green bell pepper HPL, cloned in the yeast *Y. lipolytica* was reported [21]. These values are in agreement with those reported in literature for the HPL from green bell pepper cloned in the bacteria E. coli, using immunoblot analysis [3] and SDS-polyacrylamide gel electrophoresis [22], with a molecular weight of 55 kDa.

The secondary structure analysis of the green bell pepper HPL by ANTHEPROT software was investigated (Fig. 1). These predictions were supported by three methods, the GOR I (Garnier, J. Osguthorpe, D.J. and Robson, B. Algorithm) the DPM (double prediction method) and the Predator method [18]. The GOR I predictive method is based on parameters which have been determined onto a subset of 26 proteins with known 3D structures providing structure predictions suitable for peptide segments fixed in a tertiary structure within the protein context. The DPM method consists of four successive steps: (i) prediction of the structural class of a protein from amino acids composition, (ii) preliminary secondary structure estimation from a simple algorithm, (iii) comparison between the two independent predictions, (iv) optimization of parameters and re-prediction of secondary structure. Finally, the predator method is based on recognition of potentially hydrogen-bonded residues in the amino acid sequence.

The analysis of the sequence of the green bell pepper HPL by DPM method showed 26% in α-helix. GOR and Predator methods showed higher percentages, with 39 and 34%, respectively. For β-sheet, GOR, DPM and predator methods showed similar results, 17, 21 and 16%, respectively (Fig. 1). The values calculated by these three predictive methods for the secondary structure of HPL are in agreement with those reported in literature for cytochrome P450 enzymes. The analysis of X-ray intensity of six crystal structures of cytochrome P450 (five soluble cytochrome P450 enzymes and one eukaryotic microsomal P450) showed that they have a triangular shape and are rich in secondary structure, where the percentages of α-helix and β-sheet domains are 40–70% and 10–22%, respectively [23–25].

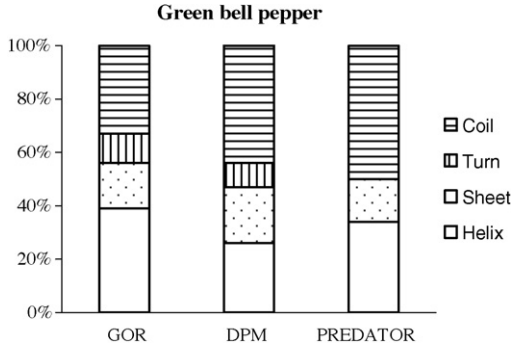

Fig. 1. Secondary structure prediction of the green bell pepper HPL.

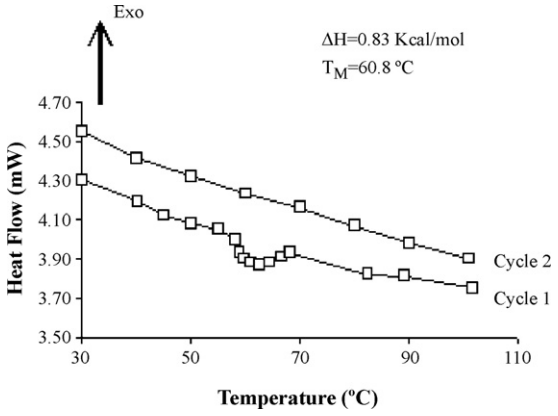

Fig. 2. DSC thermograms of purified green bell pepper HPL, expressed by the yeast *Y. lipolytica*.

3.3. Differential scanning calorimetry (DSC)

In order to determine the structural folding of the purified HPL, cloned in the yeast *Y. lipolytica*, differential scanning calorimetry (DSC) was carried out. A thermogram of HPL (pH 5.5), heated over a temperature range of 30 to 110 °C, was recorded (Fig. 2). A single endothermic peak was obtained at 60 °C, which corresponds to the denaturation temperature (T_d) with a free energy of transition ($\Delta_d H$) of 0.836 kcal/mol. These results indicate that the protein was folded after purification. In addition, a second thermal analysis cycle from 30 to 110 °C was carried out on the same sample; the experimental results showed that the denaturation of the

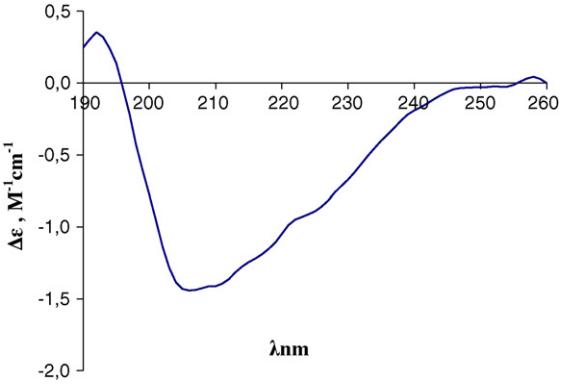

Fig. 3. CD spectra of purified green bell pepper HPL (5 μM), expressed by the yeast *Y. lipolytica*.

Table 2
Secondary structure composition of cytochrome P450 enzymes [23–25], green bell pepper HPL expressed by *E. coli* [15], and green bell pepper HPL expressed by the yeast *Y. lipolytica*, as compared to the secondary structure predictions by the ANTHEPROT software.

	Cytochrome P450 enzymes [23–25]	Bell pepper HPL		
		HPL expressed by the bacteria *E. coli* [15]	HPL expressed by the yeast *Y. lipolytica*	ANTHEPROT software
α-Helix	40–70	75	13	33
β-Sheet	10–22	–	29	18
Turn	–	–	5	7
Coil	–	–	53	42

enzyme at 60 °C was irreversible, since there was no change in the heat capacity during this second heating cycle. Indeed, the literature indicates little information on the thermal stability of P450 enzymes.

3.4. Circular dichroism analysis (CD)

The CD spectrum of purified HPL, expressed by the yeast *Y. lipolytica*, showed a small positive signal at 193 nm and two negative signals at 208 and 222 nm, respectively, which are typical characteristics of an α-helix structured protein (Fig. 3). This CD spectrum showed that the enzyme is effectively folded and confirmed the experimental results obtained by DSC. The CD spectrum was analyzed by DICROPROT V2.5 version 5.0 package [19], obtained from www.ibcp.fr, which calculates the structural composition of the enzyme; 13% α-helix, 29% β-sheet, 5% turn and 53% random coil (Table 1). The CD experimental data seem to indicate that through the different methods used for the prediction of the secondary structure of the enzyme, the ANTHEPROT analysis made with DPM calculation was the most reliable one, with 26% α-helix and 21% β-sheet. The differences between the secondary prediction and experimental data (CD) could be due to certain parameters, such as enzyme concentration, buffer or salt conditions, where the calculation cannot evaluate. Indeed, HPL requires the detergent Triton X-100 mostly for its solubilization; moreover, it has been previously reported in literature that the presence or absence of this detergent leads to remarkable change in spectral properties of HPL. The spin equilibrium of HPL moves from high spin towards low spin, when Triton X-100 is removed; this shift is reversible with the re-addition of Triton X-100 [15]. Moreover, the HPL activity is enhanced about twofold in the presence of Triton X-100; this could indicate, but without changing the global structure of HPL, that this reagent may affects the conformation of the heme site in the enzyme, which is essential for its activity.

The results obtained in the present study indicate that green bell pepper HPL, cloned in *Y. lipolytica*, contains a major percentage of β-sheet (29%) and low percentage of α-helix (13%). The secondary structure could be comparable from that previously described for some purified cytochrome P450 enzymes [23–25], especially for β-sheet (10–22%) but quite different for α-helix (40–70%). It is also different from that reported for the HPL from alfalfa [15], expressed in the bacteria *E. coli* (prokaryotic organism), which show 75% of α-helix. However, as the authors are aware it is the first time that the structural conformation of green bell pepper HPL, cloned in the eukaryotic cell of the yeast *Y. lipolytica*, is reported (Table 2).

4. Conclusion and perspectives

The analyses by bioinformatics and CD spectroscopy done in this work provide evidence to support the structural characterizations of cytochrome P450 enzymes. Indeed, our bioinformatic predicted secondary structure of HPL is in accordance with secondary structures of bacterial and mammalian cytochrome P450 enzymes [23,24] obtained by christallography. This pattern of configuration appears to be a common feature of all the enzymes of this family. However, the experimental results gathered in this study showed that HPL, cloned in the yeast *Y. lipolytica* JMY 861, has a secondary structure formed by a low percentage of α-helix (13%), a percentage of β-sheet which is quite high (29%). These structural differences in the experimental findings and predicted results could be explained by the purification conditions which requires use of detergent which can induced structural and conformational changes of the enzyme, and also may be by the fact that the HPL was tagged with a 6-histidine tail for easier purification procedure. Thus, it will be interesting to compare these structural experimental results obtained by circular dichroism combined with another HPL purification procedure. This information will be of value in determining structure/function relationships of these important class of enzymes.

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