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Secondary structure conformation of hydroperoxide lyase from green bell pepper, cloned in *Yarrowia lipolytica*, and its activity in selected media

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Abstract

Circular dichroism (CD) spectroscopy of secondary structure conformation of the purified green bell pepper hydroperoxide lyase (HPL), cloned in the yeast *Yarrowia lipolytica*, was investigated. The CD spectra of HPL in *iso*-octane, obtained at 60 °C, in the presence of the reducing agent dithiothreitol showed dramatic increase in α-helix content. The enzyme conformation remained unchanged over a range of pH values of 5.0–7.0. Using 13-hydroperoxide of linoleic acid (13-HPOD) as substrate, the biocatalysis of HPL in organic solvent media, including chloroform, dichloromethane, hexane, *iso*-octane, octane and toluene, was investigated. The results indicated an increase in HPL activity in the biphasic hexane medium.

Keywords: Hydroperoxide lyase; Secondary structure; Dichroism circular spectroscopy; Biocatalysis

1. Introduction

Hydroperoxide lyase (HPL, E.C. 4.1.2.), an enzyme widely found in plants and microorganisms, is involved in the biosynthesis of volatile aldehydes and alcohols. It catalyzes the breakdown of fatty acid hydroperoxides into oxoacids and aldehydes (C6 and C9), which are recognized as the fresh green odor of fruits and vegetables. Plant HPL is the major enzyme in the biosynthesis of natural volatile aldehydes; however, the recovery of the enzyme for its use in industrial applications is difficult [1]. The recombinant expression of the biocatalyst is the preferred way to obtain high quantities of stable and efficient enzyme [2].

The biocatalysis of enzymes in organic solvent media (OSM) is a useful approach when one or more components of the enzymatic reactions are poorly water-soluble [3]. Recently, the HPL activity was investigated in the presence of OSM, including hexane, methyl *tert*-butyl ether and acetonitrile, showing HPL activity in hexane and methyl *tert*-butyl ether, but not in acetonitrile [4]. The biocatalysis of purified soybean lipoxygenase (LOX), using linoleic acid as a model substrate, was also investigated in selected OSM, including chloroform, dichloromethane, hexane, *iso*-octane, octane and toluene [5]; the results indicated that there was an increase of 2.6-fold in LOX activity in the monophasic *iso*-octane medium compared to that obtained in the aqueous one. In addition, the experimental data obtained in these studies showed that the presence of a small volume of an organic solvent was necessary to enhance the enzymatic activity [5,6]; the same authors reported that several approaches have been used to investigate the enzymatic behavior in water-restricted environment, including the use of water miscible organic solvent systems [7,8], biphasic aqueous-organic solvent systems [9,10], reversed micelle systems [11] and monophasic organic solvent systems [12].

Circular dichroism (CD) spectra of HPL from alfalfa, cloned in the bacteria *Escherichia coli*, showed 75% of α-helix and there was no obvious structural differences in the presence or in the absence of 0.2% Triton X-100 [13]. In addition, CD studies at different temperature, pH and reaction media can be used for

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providing information on the stability of the proteins [14]. CD is also an effective tool in monitoring the changes in the secondary and tertiary structures of protein binding [15,16].

The objective of this research was to study the conformation of the secondary structure of HPL from green bell pepper cloned in the yeast *Yarrowia lipolytica,* by CD spectroscopy, at different conditions of enzymatic reaction. In addition, the HPL activity and its secondary conformation in OSM at different temperatures and pH values were investigated.

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 HPO lyase was previously constructed by Bourel et al. [17]. It 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 100 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 for its use as source of HPL.

2.2. HPL extraction and purification

All steps of extraction and recovery were carried out at 4 °C, unless otherwise indicated. The biomass of the yeast was washed three times with Tris–HCl buffer (100 mM, pH 8.0). The washed cells were suspended in the same buffer, but also containing 2% Triton X-100R (for protein extraction) (Sigma Chemical Co.; St-Louis, MO, USA), 0.3 M NaCl, 2 mM imidazole and EDTA-free protease Inhibitor Cocktail Tablets (Roche Diagnostics, Penzberg, Germany), using one tablet per 50 mL of the Tris–HCl buffer or 1 g per 6 mL buffer. The cell suspension was homogenized using a one-shot cell disrupter (Z plus series "One shot") at 1.6 kbar. The homogenized disrupted cell suspension was centrifuged (10,000 × *g*, 20 min) three times and the supernatant was considered to be the crude enzymatic extract.

Three millilitres of the resin Ni-NTA Agarose (QUIAGEN, Courtaboeuf, France) was preliminary equilibrated on column ($d = 1$ cm, $h = 1$ cm) with 15 mL of buffer A (Tris–HCl, 100 mM, pH 8.0), containing 0.2% Triton X-100R, 0.3 M NaCl and 2 mM imidazole, and at a flow rate of 0.5 mL/min. Thirty millilitres of crude enzymatic extract were loaded onto the equilibrated resin at a flow rate of 0.3 mL/min. The resin was then washed with 15 mL of buffer A and 15 mL of buffer B (sodium phosphate, 50 mM, pH 6.0), containing 0.2% Triton X-100R, 0.3 M NaCl and, 15 mM imidazole, and at a flow rate of 0.5 mL/min. The His-tagged protein was eluted with 30 mL buffer C (sodium phosphate, 50 mM, pH 5.0, containing 250 mM imidazole), dialyzed for the removal of imidazole and Triton X-100R and finally concentrated by using Microcon Centrifugal Filter devise (Millipore, France). The protein concentration was determinate using a calculated A_{280} coefficient. The numbers of Trp, Tyr and Phe residues were deduced from the HPL gene sequence, published by Matsui et al. [18].

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/mL). The biphasic organic solvent system consisted of (0–5%, v/v) of one of the selected organic solvents, including *iso*-octane, *n*-hexane, dichloromethane, octane, toluene and chloroform, in potassium phosphate buffer (100 mM, pH 5.5). 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 1 min.

2.4. 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 DICHROPROT V2.5 application package [19] to estimate the secondary structure composition. Protein samples were suspended in potassium phosphate buffer (100 mM, pH 5.5) and scanned at different temperatures (5, 20, 40 and 60 °C) or at different pH (5.0, 5.5, 6.0 and 7.0) and scanned at 20 °C. The reaction medium contained 3% of one of the selected organic solvents as well as dithiothreitol (DTT), as a reducing agent, and potassium ferricyanide, as an oxidizing agent. For the measurement, quartz Suprasil cuvettes, with a path length of 1 cm, were used. The spectra were recorded in the wavelength range of 180–260 nm, in $\Delta\varepsilon\,M^{-1}\,cm^{-1}$ for residue in function of path length λ nm. The path intensity, at 222 nm, was set in a manner to estimate the helices percentage in the polypeptide: $P\alpha = -[\ddot{A}\mathring{a}_{222} \times 10]$ ($P\alpha$: helices α percentage; $\ddot{A}\mathring{a}_{222}$: dichroic increment for residue at 222 nm) [20].

3. Results and discussion

3.1. HPL activity in organic solvent media

HPL activity was investigated in various biphasic systems, containing potassium phosphate buffer solution (100 mM, pH 5.5) and 1% of one of the selected organic solvents. For the control assays, only buffer solution was used. The results in Table 1 showed an increase in HPL activity of 1.50, 1.50 and 1.28 folds, when *iso*-octane, *n*-hexane and dichloromethane were used, respectively, in the reaction medium as compared to that obtained in the aqueous one. The maximum enzymatic activity was found in the *iso*-octane and hexane biphasic sys-