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Stability and structural changes of horseradish peroxidase: Microwave versus conventional heating treatment

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ABSTRACT

Effects of conventional heating (CH) and microwave (MW) on the structure and activity of horseradish peroxidase (HRP) in buffer solution were studied. CH incubation between 30 and 45 °C increased activity of HRP, reaching 170% of residual activity (RA) after 4–6 h at 45 °C. CH treatment at 50 and 60 °C caused HRP inactivation: RA was 5.7 and 16.7% after 12 h, respectively. Secondary and tertiary HRP structural changes were analyzed by circular dichroism (CD) and intrinsic fluorescence emission, respectively. Under CH, activation of the enzyme was attributed to conformational changes in secondary and tertiary structures. MW treatment had significant effects on the residual activity of HRP. MW treatment at 45 °C/30 W followed by CH treatment 45 °C regenerated the enzyme activity. The greatest loss in activity occurred at 60 °C/60 W/30 min (RA 16.9%); without recovery of the original activity. The inactivation of MW-treated HRP was related to the loss of tertiary structure, indicating changes around the tryptophan environment.

1. Introduction

Peroxidases (POD) are heme proteins involved in the oxidation of a wide range of organic and inorganic substrates by either H_2O_2 or organic peroxides as terminal oxidants. They have been found in multiple isoforms in several fruits and vegetables. Peroxidases are well-characterized enzymes and numerous studies have been carried out with the isoform from horseradish roots (*Armoracia rusticana*). Horseradish peroxidase (HRP EC 1.11.1.7) has several applications, such as in coupled enzyme assay in organic synthesis as a coupled enzyme assay, in chemiluminescence assays, immunoassays, and wastewater treatment and bioremediation [1]. However, their industrial application in organic synthesis, as is the case of most enzymes, has been limited due to their low thermostability and low activity in organic solvents, the ideal media for solubilization of hydrophobic substrates [2]. In this direction, some studies have been presented in the literature regarding the utilization of alternative media for biocatalysis, such as: supercritical fluids (SCF) [3,4], ionic liquids (IL) [5], and the use of microwave irradiation [6].

In contrast, peroxidases are in general not desirable in the food industry, due to browning of fruits and vegetables. This is a major problem in the food industry and is considered to be one of the main causes of quality loss during post harvest handling and processing. By rapid degradation of anthocyanins caused by PPO (polyphenol oxidases) and POD, producing brown by-products. Researchers have reported that increase in POD activity enhances enzymatic browning during storage [7]. Therefore, in foods, the extent of POD inactivation is a major index of their quality. Since enzymatic browning reaction of the products usually results in a negative influence on food quality, preventing or inhibiting enzymatic browning has become a significant tool for improving food quality during food processing.

In recent years, in order to maintain the original characteristics of foods and to reduce production costs, alternative methods to conventional thermal heating have been investigated as potential

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technologies to inactivate vegetable POD, such as pulsed electric field [8], ultrasound [9–11], supercritical carbon dioxide ($SCCO_2$) [12], UV radiation pre-treatment [13] and microwave irradiation [14–17].

Microwave irradiation (MW) offers a clean, inexpensive, and convenient method of heating, which is an alternative way to provide energy to chemical systems. Microwave effects in chemical reactions are related to the short-range molecular friction, due to continuous polarization of molecules caused by microwave irradiation. This process of ordered (with electromagnetic field) and disordered (without the field) dipoles, enhances the molecular attrition increasing the local temperature and reaction rates [6]. The heating of liquids using microwaves can be explained by the interaction of matter with the electromagnetic field of the incident radiation, causing the movement of ions as well as that of induced or permanent dipoles. The movement of such species causes heat generation (the so-called dielectric heating) by two main mechanisms: dipole rotation, which is related to the alignment of molecules that have permanent or induced dipoles with the electric field component of the radiation, and ionic conduction, which refers to the migration of dissolved ions with the oscillating electromagnetic field. Besides the quick heating of the materials, some authors consider that the microwaves can provide specific effects (not purely thermal) generally connected to the selective absorption of microwave energy by polar molecules [18]. According to this concept, microwave irradiation results in a change of thermodynamic properties of the reactive systems. An example of this effect would be the reduction of Gibbs free energy of activation of reactions due to the storage of microwave energy as vibrational energy of a molecule or functional group (enthalpic effect), or by the alignment of the molecules (entropic effect) [19]. In addition, it is believed that microwaves favor the efficiency of molecular collisions, due to the orientation of polar molecules involved in the reaction. These properties make the microwave irradiation an interesting tool for application in enzymatic processes, including inactivation of peroxidases.

Its well-known fact that during dielectric heating, radiation penetrates the material so that heat transfer takes place from the center of the material to the surface. This type of transfer causes heating of the bulk material and a rapid increase in its temperature [20]. A striking feature of the dielectric heating is its selectivity for certain types of material, especially for those with polar characteristics and high dielectric constant (a parameter that denotes the capability to store electromagnetic energy), as aqueous solutions of glucose or others sugars and ionic compounds, for example. In the particular case of mixtures, preferential heating of certain components can result in the formation of hot spots within the sample, that is, regions where the temperature is well above the mean sample temperature [20]. Regarding to the effects of MW on POD stability, some works were developed focusing on the enzyme inactivation. On the other hand, there is a lack of information about conformational changes of the enzyme caused by MW irradiation.

Horseradish peroxidase (HRP) is a typical, widely studied representative of class III heme peroxidases, and its structure and biochemical properties have been well characterized [21]. The C isoenzyme of HRP is a monomeric glycoprotein with a molecular mass of ~44 kDa. This protein contains a prosthetic heme group buried in the central region of the protein [22]. Moreover, this enzyme contains four disulphide bridges and binds two Ca^{2+} ions, proximal and distal to the heme group, that do not directly participate in the catalyzed reactions, but play an important role in sustaining the protein structure, thus assuring the enzymatic activity of HRP [23].

The main secondary structure element of this enzyme is the α-helix. Crystallographic studies showed that 44% of amino acids residues are arranged in 12 α-helices and 2% in β-sheets [24]. The HRP C isoenzyme contains a single tryptophan (Trp117), allowing to investigate conformational changes by fluorescence techniques. The intrinsic fluorescence emission of tryptophan is extremely sensitive to changes in its microenvironment [25]. The intensity of emission is related to the protein conformation, which may expose or burry the internal tryptophan residues. In HRP like other heme-containing enzymes, due to the intramolecular tryptophan-heme energy transfer, the intrinsic fluorescence emission from tryptophan residues is quenched [26]. Conformational changes and denaturation increases the distance between the tryptophan residue and the heme group. As a result, the heme quenching effect of tryptophan emission is weakened and the fluorescence intensity increases [21,27].

Thus, the changes in the structure of the heme cavity affecting the distance/orientation between the heme and the tryptophan can affect the intrinsic fluorescence of HRP. Furthermore, the change in the microenvironment surrounding the Trp residue (Trp117) can alter the emission maximum of the tryptophan fluorescence [21]. The intrinsic fluorescence of HRP is greatly dependent on the fluorescence energy transfer from tryptophan to heme [21,27]. The far UV-CD (190–250 nm) and tryptophan fluorescence emission spectrum provide information about the secondary and tertiary structure of the protein [28].

The present work is focused on the investigation of the effects of MW and conventional heating on the structure and activity of HRP. For this purpose, HRP was selected as a model enzyme and the influence of MW and conventional treatments on the residual enzyme activity and on the enzyme conformation was investigated using far UV-CD and fluorescence spectroscopic techniques.

2. Experimental procedures

2.1. Chemicals

HRP isoenzyme C was purchased from Sigma (RZ = 3.0). Guaiacol and hydrogen peroxide 30% substrates were obtained from Reagen and Vetec, respectively.

2.2. Preparation of the HRP solution

The HRP solution was prepared by dissolving 15.9 mg of the powder in 25 mL of phosphate buffer – $Na_2HPO_4{\cdot}2H_2O$-100 mM pH 6.0 (0.636 mg powder/mL). The HRP concentration for MW and conventional heating experiments was 15.9 μM, considering a molecular mass of 44 kDa [22]. The spectroscopic analyses were done at 5.3 μM.

2.3. Activity measurement

Peroxidase enzymatic activity was determined by a colorimetric method, based on the change of absorbance at 470 nm due to the formation of tetraguaiacol (product of the guaiacol oxidation) [3,4]. Peroxidase assay medium contained phosphate buffer 100 mM (pH 6.0); enzyme preparation, guaiacol 100 mM, and 2.0 mM H_2O_2, at 25 °C. One unit of enzyme [U] was defined as the quantity of enzyme enough to produce 1 μmol of the product in 1 min. The activities were expressed in U/mL. The reaction was followed in a UV–vis spectrophotometer (U-180, Hitachi, Tokyo, Japan). Control experiments (blank runs) were always carried out using the same procedure, but in the absence of peroxidase.

The residual activity (RA) of HRP was obtained with the following expression, where CH and MW denote the conventional and microwave heating treatment, respectively.

$$\mathrm{RA}(\%) = \frac{\text{activity of HRP after treatment (CH or MW)}}{\text{activity of HRP before treatment (CH or MW)}} \times 100$$

2.4. Apparatus and conventional heating (CH) procedure

HRP solution (255.6 ± 1.3 U/mL) (3.0 mL) was placed in a hermetically sealed tube and immersed in a thermostatic bath (Nova Tecnica, Piracicaba, Brazil). The heating time was measured after the solution temperature reached the desired (30, 40, 45, 50 and 60 °C).

This temperature range was chosen to favor maximum enzyme activity, aiming, for example, its use in organic synthesis. Besides inactivation of the enzyme with maximum efficiency at the lowest temperatures, is interesting for the food industry. After incubation, at established times, samples of 0.4 mL were collected and then 0.1 mL was used for the enzyme activity determination and the remainder volume