LWT - Food Science and Technology 54 (2013) 57–62

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Biochemical characterization and thermal inactivation of polyphenol oxidase from radish (*Raphanus sativus* var. *sativus*)

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ARTICLE INFO

Article history:
Received 5 December 2012
Received in revised form
10 April 2013
Accepted 16 April 2013

Keywords:
Enzymatic kinetics
Thermal stability
Minimally processing
Polyphenoloxidase
Radish

ABSTRACT

Polyphenoloxidase (PPO) is the target for the development of several food antibrowning agents. Different substrates (pyrocatechol, gallic acid, chlorogenic acid, caffeic acid, 3,4 dihydroxybenzoic acid, p-cumaric acid, L-tyrosine, pyrogallic acid and phloroglucinol) were analyzed to determine their affinities with radish PPO. Pyrocatechol, gallic acid and pyrogallic acid were the substrates that showed high affinity based on V_{max}/K_m ratio. The optimum pH for the PPO using these three substrates were pH = 7 and the optimum temperatures were 20, 60 and 20–40 °C for pyrogallic acid, gallic acid and pyrocatechol, respectively. The kinetics of thermal inactivation was successfully modeled by a biphasic model ($r^2 > 0.888$), attributed to the presence of two enzyme fractions, a heat-labile easily inactivated even at low blanching temperatures, and a heat-resistant fraction that requires blanching temperatures above 80 °C to reach 70% of inactivation. The kinetics constants of this model for both heat-labile and heat-resistant increased with temperature in the range from 60 to 90 °C. The activation energy ratio of resistant to labile fraction was found to be 6 (E_{aL} = 142 kJ/mol).

1. Introduction

Radish (*Raphanus sativus* L.), which belongs to the Brassicaceae family, is a root crop pungent or sweet in taste with a lot of juice. Roots have variable shape and skin color, but the round, red-skinned variety is the best know (Herman-Lara et al., 2012). Radishes offer many health and nutritional benefits. They are rich in folic acid, Vitamin C and anthocyanins (Patil, Madhusudhan, Ravindra Babu, & Raghavarao, 2009). Epidemiologic evidence has suggested that diets rich in vegetables are associated with reduced risk of several diseases due to potent antioxidant properties of phytochemicals decreasing oxidative stress in consumers (Zhang et al., 2013). Although radishes are widely used in salad preparations, the rapid deterioration mainly due to slices browning decreases the marketability of these preparations. The marketing of fresh-cut salads is limited by a short shelf-life and rapid deterioration of their components due to tissue damage by slicing and similar methods of preparation (Spagna, Barbagallo, Chisari, & Branca, 2005). Gonzalez Aguilar (2001) reported for radish slices that the combination of 4-hexylresorcinol (0.001 g/L), potassium sorbate (0.05 g/L) and N-acetylcysteine (0.025 g/L) was most effective in preventing browning and deterioration for up to 18 days at 10 °C. Technological strategies for enzymatic browning inhibition should be focused on the enzyme responsible for plant tissue browning. The undesirable browning of damaged tissues in fruits and vegetables occurs by the enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenoloxidase (EC 1.10.3.1: 0-diphenol: oxygen oxidoreductase, PPO). Characterization of radish PPO is important to identify its biochemical properties and function and, in turn, to understand how to prevent its deteriorative action during storage and processing. Many studies have investigated PPO with the goal of preventing this discoloration (Quieroz, Mendes Lopes, Fialho & Valente-Mesquita, 2008; Yoruk & Marshall, 2003). Andi et al. (2011) reported the purification and characterization of polyphenols oxidase from Japanese radish root, which is a white radish; however this radish belongs to a Japanese variety namely var. L. cv. *Aokubi soufuto*-L. The most consumed variety of radish in Argentine is red radish, (*Raphanus sativus* var. *sativus*) and for this variety a characterization of the PPO has not been previously conducted.

The aims of this research were to (1) biochemical characterize the PPO of radish by determining several selected substrates specificity, (2) determine their enzyme kinetic parameters by

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http://dx.doi.org/10.1016/j.lwt.2013.04.014

mathematical modeling, (3) determine the effects of pH and temperature on the enzyme activity in order to find optimal ranges of work, (4) determine the thermal stability of the enzyme, and (5) determine the kinetics of thermal inactivation during blanching in the range of 60–90 °C by means of a biphasic model.

2. Materials and methods

2.1. Plant material and sample preparation

Radishes were purchased from a local market from Mar del Plata city. They were kept at 5 ± 1 °C in darkness prior to processing. Radish roots were separated from leaves and they were washed in tap water to eliminate any surface contamination, cut with a manual cutter into slices of 3–4 mm, and then washed again in tap water, using a ratio of sliced radish to water of 1:10 (g:g).

2.2. Measurement of the enzyme activity

The activity of PPO was measured by the colorimetric method. 10 g of radishes were homogenized at a 1:2 (g:mL) ratio with 0.5 mol/L phosphate buffer (pH = 7.0) in the presence of 50 g/L polyvinylpyrrolidone (ICN Biomedicals, Inc. OH) with a commercial mixer and centrifuged at 12,700 × g for 30 min. The supernatant, which contained PPO activity, was used as the experiment enzyme source (PPO crude vegetable extract). Crude extract was maintained at 0 °C until use. The reaction cuvette contained 2.9 mL of substrate (concentrations range from 2 to 40 mmol/L) mixture and 0.1 mL PPO crude vegetable extract. The enzyme activity was defined as a 0.001 change in absorbance between 0 and 60 s under the assay conditions, according to previous experiments. Each solution was tested in triplicate. The reference cuvette contained distillated water.

2.3. Kinetic data analysis and substrate specificity

The specificity of radish PPO extract was investigated for nine commercial grade substrates (pyrocatechol, gallic acid, chlorogenic acid, caffeic acid, 3,4 dihydroxybenzoic acid, p-cumaric acid, L-tyrosine, pyrogallic acid and phloroglucinol) at different concentrations. PPO activity was assayed in triplicate. The activity of PPO extract as a function of the substrates concentration was investigated in order to determine the enzyme kinetics. Michaelis–Menten constant (K_m) and maximum rate for the enzymatic reaction (V_{max}) were determined by means of Lineweaver–Burk method (Erat, Sakiroglu, & Kufrevioglu, 2006).

2.4. Effect of pH on enzyme activity

The activity of PPO was measured at room temperature in 0.1 mol/L acetic acid/0.1 mol/L sodium acetate in the pH range of 3.0–6.0, in 0.1 mol/L disodium hydrogen phosphate/0.1 mol/L hydrochloric acid in the pH range of 7.0–9.0 and also in 0.1 mol/L disodium hydrogen phosphate/0.1 mol/L sodium hydroxide in the pH range of 10.0–11.0. The optimum pH for the PPO was obtained using three substrates: 28 mmol/L pyrocatechol, 4 mmol/L gallic acid and 6 mmol/L pyrogallic acid. The pH value corresponding to the highest enzyme activity was taken as the optimal pH and the enzyme activity was expressed as the percentage of maximum activity speed at 25 °C.

2.5. Effect of temperature on enzyme activity

The temperature effect on the activity of radish PPO was investigated by equilibrating the substrate in a water bath (0–70 °C, at intervals of 10 °C) for 10 min before introducing the enzyme at a pH = 7. The optimum temperature for the substrates was obtained using three of them: 28 mmol/L pyrocatechol, 4 mmol/L gallic acid and 6 mmol/L pyrogallic acid. The enzyme activity was expressed as the percentage of maximum activity speed.

2.6. Thermal stability

The thermal stability of radish was investigated at optimal substrate pH, at intervals of 10 °C, from 0 to 80 °C using an incubation time of 10 min. The remaining activity of PPO was measured under the standard conditions ($T = 30$ °C). Relative PPO activity was measured using the K_m concentration of each substrate. The enzyme activity was expressed as the percentage of maximum activity speed.

2.7. Kinetics analysis of enzyme inactivation

The fist order biphasic model proposed by Fante and Zapata Noreña (2012) was used to describe the kinetics of the heat inactivation of the PPO. The mathematical expression of the model is:

$$RA = a_L \exp(-k_1 * t) + b_R \exp(-k_2 * t) \quad (1)$$

Where RA represents the value of the residual enzyme activity, k_1 and k_2 are the velocity constants of the heat labile and heat resistant components, respectively, a_L and b_R are the initial concentrations of the labile and resistant components, respectively, and t is the immersion time.

The dependence of the rate constants with temperature was assumed to follow the Arrhenius Law (Jakób et al., 2010):

$$k = k_0 \exp\left(\frac{-E_a}{RT}\right) \quad (2)$$

Where E_a is the activation energy, k_0 is the pre-exponential factor, and T is the absolute temperature.

2.8. Estimation of model parameters

Model parameters of biphasic model were estimated from the mean experimental values for each set of experimental conditions using nonlinear least-squares routines applying the function *lsqcurvefit* of the program Matlab 7.7.

2.9. Statistical analysis

Experiments were performed in triplicate. Values are expressed as means ± standard deviations. One way ANOVA (at the level of significance $P < 0.05$) was performed to ascertain the significance of the means. Statistical analysis was performed using SAS program (software version 8.0, SAS 1999).

3. Results and discussion

3.1. Substrate specificity

Phenolic compounds are the primary substrates of PPO (Yoruk & Marshall, 2003). Radish PPO showed activity with monophenolic substrate (L-tyrosine), diphenols (caffeic acid, pyrocatechol) and polyphenolics (chlorogenic acid, gallic acid, pyrogallic acid) (Table 1). p-cumaric acid (monophenol), 3,4-dihidroxibenzoic acid (diphenol) and phloroglucinol (triphenol substrate), showed no specificity for the enzyme. Probably with these last substrates the spatial orientation of the hydroxyl groups prevents enzyme and