Kinetic characterization, thermal and pH inactivation study of peroxidase and pectin methylesterase from tomato (*Solanum betaceum*)

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Abstract

Peroxidase (POD) and pectin methylesterase (PME) from tomato were characterized, studied thermal stability, and analyzed the synergistic effect of temperature and pH. For POD, the optimal activity, using H_2O_2 as substrate and $ABTS^{+\bullet}$ as the donor H^+, was obtained at pH 3.5, and for PME, the optimal activity using pectin as substrate was obtained at pH 7.5. In POD, it was found that the values of K_M, V_m and K_{si} for H_2O_2 were 477.26 mM, 721.53 µM/min and 0.37 mM, respectively. In PME, the values of K_M and V_m obtained for pectin were 0.54 mM and 436.12 µM/min, respectively. On the other hand, it was found that POD was inactivated with 90 °C, at pH from 2.5 to 3.5 with temperatures of 55 to 90 °C, and at pH of 2.5 to 3 with temperatures of 40 to 90 °C. Likewise, PME was inactivated at 90 °C, and at pH of 3.5 with 70 °C.

Keywords: peroxidase; pectin methylesterase; kinetic parameters; thermal inactivation and pH inactivation.

Practical Application: Control of the quality of the tomato for the use in the industrial processing.

1 Introduction

The tomato (*Solanum betaceum*) is a small shrub, belonging to the family of the solanaceous (Do-Nascimento et al., 2013). The fruit presents a great potential for the market due to its nutritional and organoleptic characteristics (Andrade et al., 2013; Márquez et al., 2007; Lagos et al., 2013), being fundamental for the juice and vegetable beverage industry (Rodríguez et al., 2011). The new consumers demand them instead of the natural ones for the new rhythm of life and because they maintain the nutritional characteristics of the fruit and can be easily found in the market (Cerón et al., 2016). Villareal et al. (2013) they expose that agroindustrial transformation has focused on obtaining juices and vegetable beverages.

Browning damage and loss of stability caused in fruit and vegetable tissues during postharvest treatment and processing is one of the main causes of loss of quality in juices and vegetable beverages. Peroxidase and pectin metilesterasa, responsible for a multitude of alterations in the parameters organoleptic of fruits and vegetables (Anthon et al., 2002; Núñez-Delicado et al., 2007), may be associated with losses in color, odor, taste, texture and nutritional values in juices and vegetable beverages (Cabanes et al., 2007).

To prevent undesirable enzymatic reactions, heat treatment and treatment by pH, which inactivates enzymes and helps microbiological safety, is commonly used for the conservation of many foods such as juices and vegetable beverages. However, the application of heat treatment is limited by alterations in sensory characteristics and loss of nutrients that can be caused by this process (Núñez-Delicado et al., 2003). Peroxidase and pectin methylesteraseare the thermally resistant enzymes in vegetables

and, for this reason, are generally used as indicators for the treatment and processing of food. Consequently, peroxidase and pectin methylesterase have been widely used as biological indicators of the effectiveness of browning (Chazarra et al., 1996) and loss of stability in juices and vegetable beverages (Stoforos et al., 2002).

Peroxidase (POD; EC 1.11.1.7) are heme type proteins that contain iron (III) and protoporphyrin IX as a prosthetic group. These are a group of oxidoreductases that catalyze the reduction of peroxides, such as hydrogen peroxide, coupled with the oxidation of a variety of organic and inorganic compounds (Fortea et al., 2011). Peroxidases are involved in a wide range of physiological functions in plants, some of the functions are the biosynthesis and degradation of lignin in the cell walls, defense against pathogens and the environment, and the response to stress by heavy metals (Al-Senaidy & Ismael, 2011). Peroxidases show a wide specificity for the substrate, a characteristic that makes them useful in various industrial, analytical and biomedical applications (Chung et al., 2005; Hidalgo-Cuadrado et al., 2012; Lavery et al., 2010). Many researchers have reported on the involvement of peroxidases in browning processes that cause millions of losses in the agrifood industry, however, this peroxidase activity is limited by the availability of electron acceptor compounds, such as superoxide radicals, hydrogen peroxide and lipid peroxides (Robinson & Eskin, 1991).

Pectin methylesterases (PME, EC 3.1.1.11) are widely distributed in fruits and vegetables. They are part of a group known as pectinases, which also includes polygalacturonase (PG, EC 3.2.1.15), pectin lyase (PL, EC 4.2.2.10) or pectic

acid lyase (PAL, EC 4.2.2.2) (Pilnik & Voragen, 1991). Pectin methylesterases hydrolyzed methyl ester group of pectin molecules, gradually diminishing their degree of esterification (DE). One of the main consequences for the quality of juices and vegetable beverages is the complexation of the pectin molecule with low content of of with Ca^{2+} endogenous ions, which leads to the loss of stability in juices and vegetable beverages, being detrimental in the agroalimentary industry (Laratta et al., 1995). Another effect is the much greater susceptibility of pectins with low content of a degradation by PG endo-actuating (and PAL), a process that leads to a drastic decrease in viscosity and texture in products such as tomato puree (MacMillan & Sheiman, 1974).

The objective of this work was to extract, characterize, study the thermal stability, and synergistic effect of temperature and pH, for POD and PME in tomato puree, to study the best conditions of treatment to maximize the quality and minimize the organoleptic, nutritional and therefore economic loss induced by enzymatic degradation.

2 Materials and methods

This work has focused its efforts on the application of temperatures and pH that are currently applied in the food industry to inactivate partially or totally the enzymes involved in the deterioration of tomato. Tomato was chosen due to high enzymatic activity. At the beginning of the study, two (POD and PME) of the main enzymes involved in the degradation of the tomato were extracted and characterized kinetically. Once the optimal conditions for quantification were characterized and obtained, they were used as a model because of their high activity in tomato and incubations were made at different temperatures and temperature combinations compared to pH obtaining the best conditions for inactivation in tomato.

2.1 Enzyme source

AMC INNOVA S.L. (Murcia, Spain) kindly supplied fresh tomatoes pear variety grown until to commercial maturity. The samples were transported to the laboratory and refrigerated at 4 °C and were used as a source of POD and PME.

2.2 Reagents

All reagents were acquired in Sigma (Madrid, Spain) and were used without purification. For POD, they were used as substrates [2,2'-Azino-bis-(3-etilbenzotiazoline-sulfónico acid)] $ABTS^{+\bullet}$ and H_2O_2. Hydrogen peroxide solutions were recently prepared every day, and their concentrations were calculated using $\varepsilon_{240} = 39.4$ $M^{-1}cm^{-1}$ (Nelson & Kiesow, 1972). For PME, a solution with citrus pectin at 0.5% (w/v) was used as a substrate.

2.3 POD extraction

50 g of fresh tomatoes were weighed and homogenized in an Ultra Turrax (Ika, Staufen, Germany) for 7 minutes. The homogenate was centrifuged at 5,000 rpm for 30 minutes at 4 °C. Subsequently, the supernatant was extracted and filtered through a syringe containing the crude enzyme extract, which was stored at -80 °C until its subsequent analysis.

2.4 PME extraction

PME tomato was extracted using the method described by Hagerman & Austin (1986) with some modifications. 50 g of fresh tomatoes were weighed and homogenized in an Ultra Turrax (Ika, Staufen, Germany) for 10 minutes. This homogenate, was adjusted to pH 7.0 with NaOH 0.5 N. Subsequently, NaCl was added to a final concentration of 0.5 M. The resulting mixture was centrifuged at 5,000 rpm 30 minutes at 4 °C, obtaining the supernatant. The supernatant was adjusted to pH 4.2 with HCl 0.5 N, and was filtered through a syringe with filtering obtaining the crude enzyme extract, which was stored at -80 °C until its subsequent analysis.

2.5 POD enzymatic activity

POD was determined spectrophotometrically, in a spectrophotometer Shimadzu model UV-1063, using as substrate $ABTS^{+\bullet}$ at 414 nm ($\varepsilon_{414} = 31.1$ $M^{-1}cm^{-1}$) (Rodríguez-López et al., 2000b). An enzymatic unit is the amount of enzyme that produces 1 μmol of the radical $ABTS^{+\bullet}$ per minute. The standard reaction medium at 25 °C contained, in sodium acetate buffer 100 mM, pH 3.5, 3 mM of $ABTS^{+\bullet}$, 10 mM of H_2O_2 and 20 μL of the crude enzyme extract, in a final volume of 1 mL.

2.6 PME enzymatic activity

PME was determined spectrophotometrically, in a spectrophotometer Shimadzu model UV-1063, using as a citrus pectin substrate, at 620 nm ($\varepsilon_{620} = 0.25$ $M^{-1}cm^{-1}$) (Hagerman & Austin, 1986). The assay was started at the same pH to ensure reproducibility in color changes. However, tampons interfere with the measurement of acid production, so all reagents should be prepared in very weakly buffered solutions. The standard reaction medium at 25 °C contained, in potassium phosphate buffer 3 mM at pH 7.5, 300 μL of citrus pectin 1% (w/v) (containing 0.0005 mM of NaCl), 70 mL bromothymol blue to 0.01% (w/v) and 20 μL of crude enzyme extract, in a final volume of 1 mL.

2.7 Optimum pH

For POD and PME, the optimum pH was determined in a standard reaction medium, containing in 100 mM sodium acetate buffer (pH 3.0-5.0), 100 mM sodium phosphate buffer (pH 6.0-7.5) and sodium borate buffer 100 mM (pH 8.0-9.0).

2.8 Kinetic parameters

Once the optimal pH was calculated for each enzyme, to determine the kinetic parameters in POD, the effect on the concentration of $ABTS^{+\bullet}$ and H_2O_2 for enzymatic activity was studied. The effect was studied in the concentration of $ABTS^{+\bullet}$, increasing from 0 to 5 mM in the standard reaction medium. In the case of H_2O_2, its concentration increased from 0 to 60 mM in the standard reaction medium, at a fixed concentration of $ABTS^{+\bullet}$ (3 mM). For PME, the effect on the concentration of citrus pectin was studied. To determine the effect on pectin concentration, it was increased from 0 to 40 mM in the standard reaction medium.

2.9 Thermal stability

The thermal stability in POD and PME was determined by measuring the enzymatic activity with temperatures of 20, 40, 45, 50, 55, 60, 65, 70 and 90 °C, at optimum pH. The temperature was controlled using a TCC controller, Corporación Shimadzu (Mod. CBT-240A).

2.10 Effects of temperature and time on enzyme stability

Enzymatic stability at varying temperatures and incubation times was determined, incubating aliquots with 15 ml of tomato puree extract (Eppendorf tubes) in a circulating water bath Julabo Shake Temp SW 22, with different temperatures (20, 40, 45, 50, 55, 60, 65, 70 and 90 °C) at different times (from 5 to 30 minutes, with 5 minutes intervals) for POD and PME. After heating, the samples were cooled in an ice bath and the residual activity of POD and PME was determined under the conditions that were considered optimal for each enzyme.

2.11 Effects of pH and temperature on enzyme stability

The enzymatic stability at pH and variable temperatures was determined, incubating aliquots with 15 ml of tomato puree extract (Eppendorf tubes) at pH values ranging from 2.5 to 4.5 (with intervals of 0.5) for POD and pH values varying from 3.5 to 4.5 (with intervals 0.5) for PME. Subsequently, each of these aliquots adjusted to different pH, were incubated with temperatures of 20, 40, 45, 50, 55, 60, 65, 70 and 90 °C (for 5 minutes) for POD, and with temperatures of 20, 50, 60, 70 and 90 °C (for 5 minutes) for PME. The samples were cooled in an ice bath and the residual activity of POD and PME was determined under the conditions that were considered optimal for each enzyme.

2.12 Statistical analysis

The statistical analysis was carried out, each sample was analyzed in triplicate and the mean ± standard deviation was represented.

3 Results and discussion

The purification of enzymes in plant extracts is a difficult process due to the presence of a wide variety of secondary products which can bind strongly to enzymes and change their characteristics (Chung et al., 2005). To solve this problem, different methods have been developed, without the need to use detergents. For POD, the simple use of the Ultra Turrax with the ability to break the biological membranes and to cover the soluble enzymes in the cytosol, was enough for its extraction. In addition, a centrifugation was required at 4 °C to avoid enzymatic overexpression during the centrifugation time, to obtain an enzyme rich supernatant and poor in impurities such as fibers that constitute the tomato matrix. PME could be extracted as POD, but needed pH adjustment and NaCl addition to be extracted and stored.

pH is a determining factor in the expression of enzymatic activity. Figure 1A shows the pH profile for the oxidation of ABTS$^{+\bullet}$ per POD in a pH range from 3.0 to 8.0. For POD, it was found that the optimal pH was 3.5, similar to that obtained in marula (Bomben et al., 1975) and red cabbage (Fortea et al., 2012), although lower than that obtained for red algae (pH 5.0) (Fortea et al., 2011). Figure 1B shows pH sweep for PME in a pH range of 5.0 to 8.0. For PME, it was found that the optimal pH was 7.5, similar to that obtained in apple (Denès et al., 2000), banana (Ly-Nguyen et al., 2002a), kiwi (Pal et al., 2016) or carrot (Ly-Nguyen et al., 2002b), and lower to acerola (pH 9.0) (De Assis et al., 2002).

To characterize POD and PME, the detailed study of its kinetic parameters was carried out. The apparent kinetic parameters (V_m and K_M) of POD and PME were determined by adjusting the experimental points to the Michaelis-Menten equation using the data obtained at pH 3.5 for POD, and at pH 7.5 for PME. Figure 2A shows the variation in the initial velocity against the substrate concentration (H_2O_2) for POD, and the Figure 3 shows the variation in the initial velocity against the substrate concentration (pectin) for PME.

When the concentration of H_2O_2 was increased to a fixed and saturated concentration of ABTS$^{+\bullet}$ (3 mM), it was observed an inhibition by high concentration of substrate. Figure 2A shows a kinetic profile of inhibition by the high concentration of the substrate, which can be analyzed kinetically by of the nonlinear regression adjustment of the experimental points to the following equation (Rodríguez-López et al., 2000b, Equation 1):
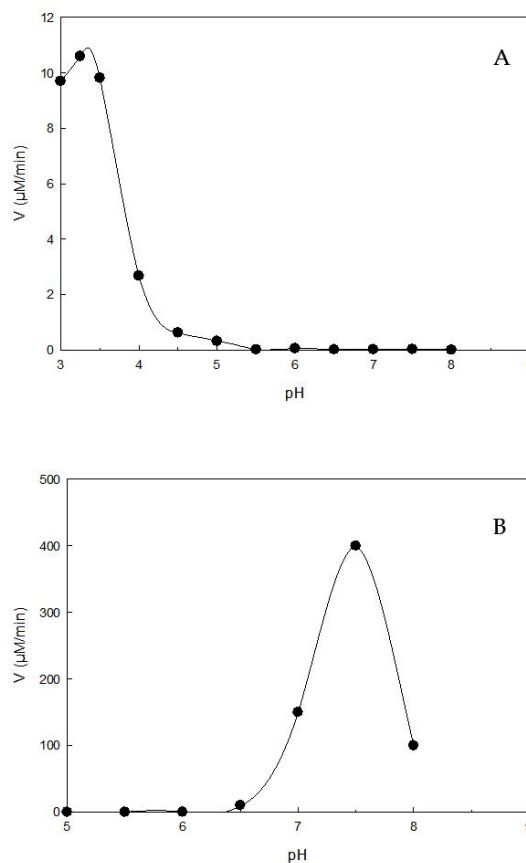
Figure 1. (A) Effect of pH for POD activity, in the presence of 10 mM of H_2O_2 and 3 mM of ABTS$^{+\bullet}$. (B) Effect of pH for PME activity, in the presence of 10 mM of citrus pectin.
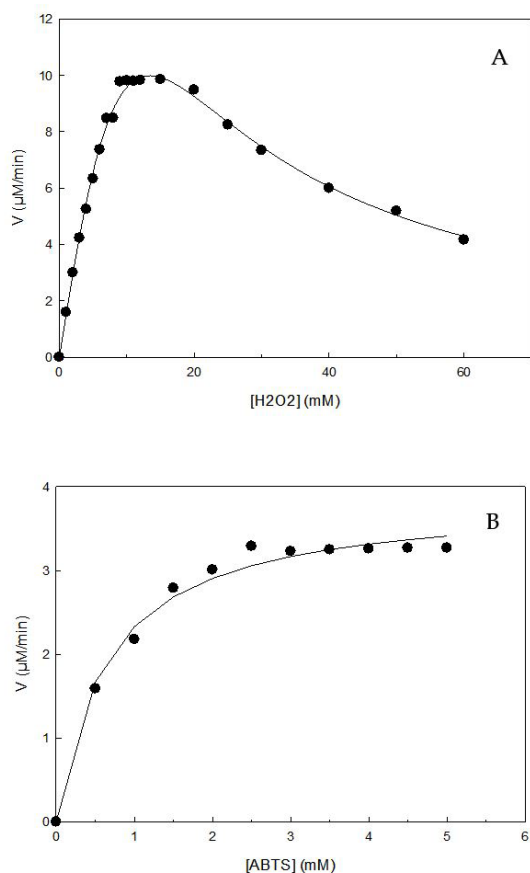
Figure 2. (A) Effect of the concentration of the H_2O_2 for POD activity. (B) Effect of the ABTS$^{+\bullet}$ concentration for POD activity.
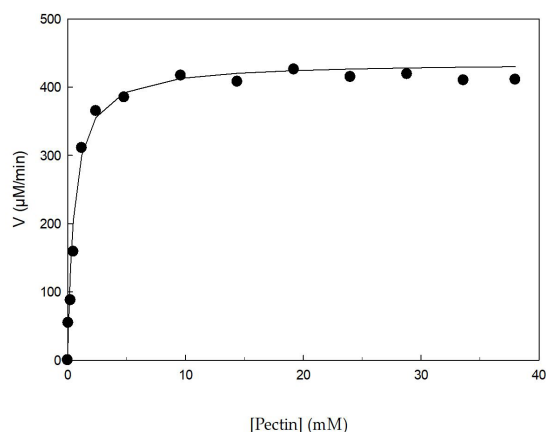
Figure 3. Effect on the concentration of citrus pectin for PME activity.

$$v = \frac{V_m[S]}{K_M + [S] + [S]^2 / K_{si}} \tag{1}$$

Where K_{si} is the substrate inhibition constant. The kinetic parameters V_m, K_M and K_{si}, were 721.53 µM/min, 477.26 mM and 0.37 mM, respectively.

This K_M value obtained for H_2O_2 was much higher than that described in red cabbage (0.98 mM) (Fortea et al., 2011), turnip (0.8 mM) (Bradford, 1976), pepper (1.3 mM) (Cabanes et al.,

2007), grape (0.4 mM) (Bordier, 1981) or melon (0.2 mM) (Bahçeci et al., 2005). The kinetic profile is consistent with the obtained for horseradish peroxidase, asparagus, turnip and red algae (Fortea et al., 2011) (Núñez-Delicado et al., 2005) describing an inhibition by high concentration of substrate in the case of H_2O_2.

Figure 2B shows the kinetic profile of ABTS$^{+\bullet}$ for POD, at a fixed concentration of H_2O_2 of 2 mM, the value of K_M was 0.65 mM and the value of V_m was 3.85 mM/min. The value of K_M for ABTS$^{+\bullet}$ was similar to that described for red cabbage (Fortea et al., 2012), turnip (Bradford, 1976), peach (Cabanes et al., 2007), and lower for horseradish (4 mM) (Núñez-Delicado et al., 2005) or red algae (13 mM) (Fortea et al., 2011). In addition, when the concentration of ABTS$^{+\bullet}$ increased from 0 to 5 mM (Figure 2B), at a fixed concentration of H_2O_2 (2 mM), the activity increased to saturationFigure 2B shows a saturation kinetic profile with high concentration of the substrate.

For PME, when the pectin concentration was increased from 0 to 38 mM (Figure 3), the activity increased until saturation was reached. The K_M and V_m value got for pectin was 0.54 mM and 436.12 µM/min, respectively. The K_M value was similar to that described in apple (Denès et al., 2000) and acerola (De Assis et al., 2002), and lower to banana (0.77 mM) (Ly-Nguyen et al., 2002a), kiwi (0.84 mM) (Pal et al., 2016) or carrot (1.01 mM) (Ly-Nguyen et al., 2002b).

A thermal stability study for POD (Figure 4A) and PME (Figure 4B) were carried out. For POD, (Figure 4A) temperatures similar or higher than 60 °C (from 5 to 30 minutes) cause a slight but gradual decrease of the activity, until an residual enzymatic activity of 60% was observed at 70 °C (from 5 minutes of incubation). For PME, (Figure 4B) temperatures similar or higher than 40 °C (from 5 minutes of incubation) caused a slight but gradual decrease in the activity, until an residual enzymatic activity of 55% was observed at 70 °C (from 5 minutes of incubation).

The temperature required for total POD inactivation was 90 °C (Figure 4A), being greater than the one described in red algae (30-50 °C) (Fortea et al., 2011), pepper (30-60 °C) (Cabanes et al., 2007), red grape (60-80 °C) (Bordier, 1981), strawberry (50-80 °C) (Hiner et al., 1996) or melon (40-70 °C) (Rodríguez-López et al., 2000a). Similarly, the temperature required for the total inactivation of PME was 90 °C (Figure 4B), being greater than the one described in banana (80 °C) (Ly-Nguyen et al., 2002a), carrot (65 °C) (Ly-Nguyen et al., 2002b), mango (78 °C) (Díaz-Cruz et al., 2016) or tomato (78,5 and 75 °C) (Anthon et al., 2002; Stoforos et al., 2002, respectively).
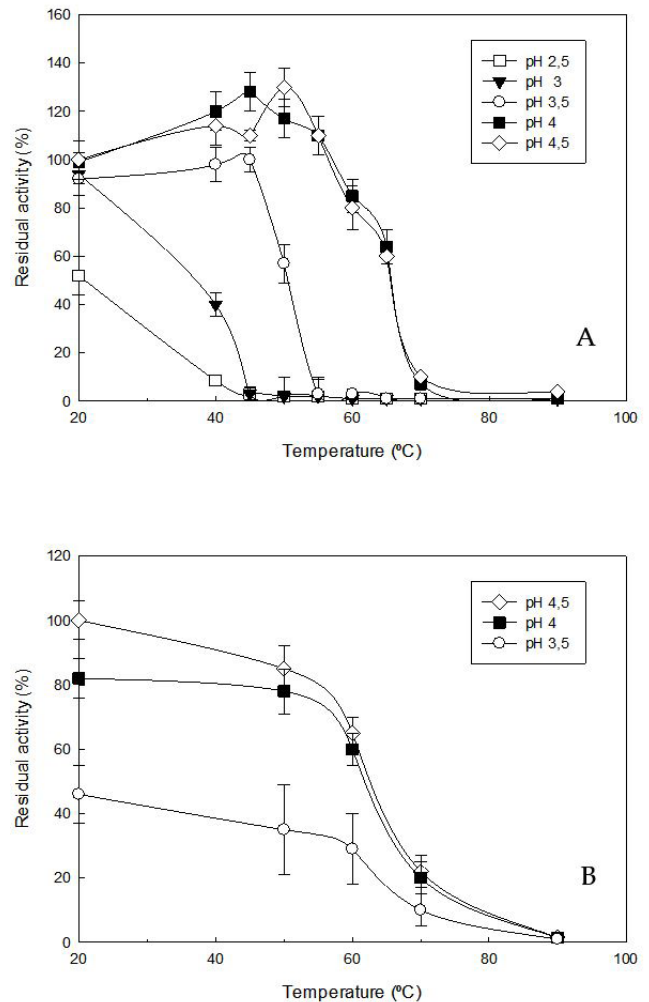
On the other hand, it was carried out the study of the synergistic effect of pH and temperature for POD and PME, observing in both enzymes a strong pH dependent activity in all temperatures studied. For POD, the extracts between 20 and 90 °C were confronted (the extracts were incubated for 5 minutes at each temperature) at a pH range of between 2.5 and 4.5 (Figure 5A). The observed enzyme activity decreased gradually when the pH was reduced and the temperature increased. At pH values between 2.5 and 3.5 with temperatures ranging from 55 to 90 °C, POD residual activity was 1-3%. On the other hand, at pH values ranging from 2.5 to 3 with temperatures of 40 to 90 °C, the residual
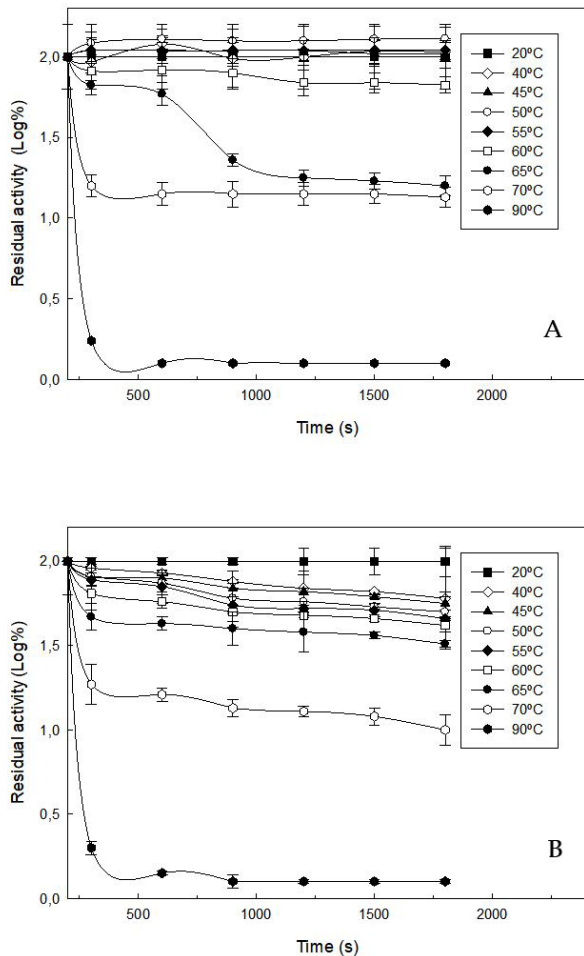
Figure 4. (A) Thermal inactivation of POD. Residual POD activity versus the heating time at 20 °C (■), 40 °C (◇), 45 °C (▲), 50 °C (○), 55 °C (◆), 60 °C (□), 65 °C (●), 70 °C (○) and 90 °C (●). (B) Thermal inactivation of PME. Residual PME activity versus to the heating time at 20 °C (■), 40 °C (◇), 45 °C (▲), 50 °C (○), 55 °C (◆), 60 °C (□), 65 °C (●), 70 °C (○) and 90 °C (●).

Figure 5. (A) Synergistic effect of pH and temperature for POD. Residual POD activity versus temperature at pH 2.5 (□), 3 (▼), 3.5 (○), 4 (■) and 4.5 (◇). (B) Synergistic effect of pH and temperature for PME. PME residual activity versus temperature at pH 3.5 (○), 4 (■) and 4.5 (◇).

activity of POD was 1-6%, being similar to that obtained in peach at pH 3 and 30 °C (Toralles et al., 2005), and less than that obtained in grape caimarona at pH 4 and 25 °C (25%) (Rivera-Camelo et al., 2004). However, we found an activation of enzyme activity at pH 4 and 4.5 with temperatures ranging from 40 to 60 °C, reaching an activation of 30% at pH 4.5 and 50 °C, being similar to that obtained in peach to pH 4 and 30 °C (Toralles et al., 2005). For PME, the extracts were confronted with temperature values ranging from 20 to 90 °C (the extracts were incubated for 5 minutes at each temperature) with a pH range of between 3.5 and 4.5 (Figure 5B). At pH values of 3.5 with temperatures of 70 and 90 °C, the residual enzymatic activity of PME was 8% and 2%, respectively, being similar to that obtained in apple at pH 4 and 35 °C (Denès et al., 2000) and less than what obtained in acerola at pH 8.3 and 98ºC (65%) (De Assis et al., 2002).

These results show that POD and PME of tomato are inhibited more effectively as it decreases the pH and increases the temperature, being especially sensitive to pH ≤ 3.5 and temperatures ≥ 70 °C.

4 Conclusion

In conclusion, this paper presents for the first time a detailed study of kinetic characterization, thermal stability and synergistic effect of temperature and pH for POD and PME in tomato puree. Inactivation of both enzymes is very important, since these enzymes can have very negative effects on the color, odor, flavor and texture of juices and vegetable beverages during storage. POD was found to be inactivated with 90 °C (for 5 minutes), at pH from 2.5 to 3.5 with temperatures from 55 to 90 °C (for 5 minutes), and at pH from 2.5 to 3 with temperatures of 40 to 90 °C (for 5 minutes). Likewise, PME was inactivated at 90 °C (for 5 minutes), and at pH of 3.5 with 70 °C (for 5 minutes). The browning and loss of stability in juices and vegetable beverages, such as tomato puree, can be controlled by applying temperature and pH combinations capable of inactivating these enzymes in a total or partial way, but while respecting the limits organoleptic and legal for juices and vegetable beverages.

Acknowledgements

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