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Kinetic characterisation and thermal inactivation study of polyphenol oxidase and peroxidase from table grape (Crimson Seedless)

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ABSTRACT

Polyphenol oxidase (PPO) and peroxidase (POD) were extracted from a table grape (Crimson Seedless) using Triton X-114 and characterized using spectrophotometric methods. Both PPO and POD were activated by acid shock. However, in the presence of the anionic detergent sodium dodecil sulphate (SDS), PPO was activated whereas POD was inactivated. The enzymes were kinetically characterized and both followed Michaelis–Menten kinetics, although with different values of their kinetic parameters. The V_m/K_m ratio showed that Crimson Seedless grape PPO presents a similar affinity for 4-*tert*-butyl-catechol (TBC) whether activated by acid shock (0.018 min^{-1}) or SDS (0.023 min^{-1}). With regards to POD, the K_m and V_m values for 2,2′-azinobis(3-ethylbenzothiazolinesulphonic acid) (ABTS) were 0.79 mM and 1.20 μM/min, respectively. In the case of H_2O_2, the K_m and V_m value were 0.4 mM and 0.93 μM/min, respectively. PPO and POD showed similar thermostability, losing >90% of relative activity after only 5 min of incubation at 78 °C and 75 °C, respectively. In addition, PPOs activation energy was similar to that obtained for POD (295.5 kJ/mol and 271.9 kJ/mol, respectively).

1. Introduction

Crimson Seedless grape is one of the most important seedless varieties due to its attractive cluster with medium-large red berries. It presents excellent taste characteristics due to its texture, firmness and flavour. Crimson Seedless grapes show good strength during post-harvest manipulation, transport and cool storage (Dokoozlian, Peacock, & Luvisi, 1993). Its main problem is poor berry colour, and while cultural practices focus on increasing yield and berry weight, the same practices tend to reduce colour intensity (Dokoozlian et al., 1993).

The browning reaction, which results from mechanical injury during post-harvest storage or the processing of fruits and vegetables, is a widespread problem. This process arises from the oxidation of phenolic compounds and contributes significantly to quality loss (Lamikanra & Watson, 2001). In general, the main enzymes responsible for the browning reaction are polyphenol oxidase and peroxidase (Gónzalez-Barrio, Salmenkallio Martilla, Tomás-Barberán, Cantos, & Espín, 2005; Mayer & Harel, 1979). Polyphenol oxidase (PPO; EC 1.14.18.1) is a copper-containing enzyme, which, in the presence of oxygen, catalyses the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity) (Orenes-Piñero, García-Carmona, & Sánchez-Ferrer, 2006). These, in turn, are polymerised to undesirable brown, red, or black pigments (Gandía-Herrero, García-Carmona, & Escribano, 2004). In plants, PPO is predominantly located in the chloroplast thylakoid membranes, and its phenolic substrates are mainly located in the vacuoles but, following any cell-damaging treatment, the enzyme and substrates may come into contact, leading to rapid oxidation of the phenols (Chazarra, García-Carmona, & Cabanes, 2001). These reactions lead to changes in the physical, chemical or nutritional characteristics of food and beverages, decreasing the quality of the final product (Martínez & Whitaker, 1995). Quinones also lead to polymerisation and condensation reactions between proteins and polyphenols, leading to the formation of brown pigments. Because of the importance of this reaction in the food industry, PPO has been intensively studied in several plant tissues such us spinach (Golbeck & Cammarata, 1981), apricot (Arslan, Temur, & Tozlu, 1998), tea leaves (Halder, Tamuli, & Bhaduri, 1998), grape (Núñez-Delicado, Sánchez-Ferrer, García-Carmona, & López-Nicolás, 2005a; Núñez-Delicado, Serrano-Mejías, Pérez-López, & López-Nicolás, 2005b, 2007), peach (Cabanes, Escribano, Gandía-Herrero, García-Carmona, & Jiménez-Atiénzar, 2007), persimmon (Núñez-Delicado, Sojo, García-Cánovas, & Sánchez-Ferrer, 2003), banana (Sojo, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 1998) and iceberg lettuce (Chazarra, Cabanes, Escribano, & García-Carmona, 1996).

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Peroxidase (POD; EC 1.11.1.7) is another oxidoreductase enzyme involved in enzymatic browning, because diphenols may function as reducing substrates in this reaction (Robinson, 1991). The involvement of POD in browning has been reported by many researchers (Richard-Forget & Gauillard, 1997), although it is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide, and lipid peroxides. Furthermore, it has been proposed that POD catalyses the cross-linking between the ferulic acid substituents of pectins (Fry, 1986), and a clear correlation has been found between its activity and the synthesis of lignin and suberin polymers. Oxidation of a wide range of organic compounds has led to speculation that this enzyme may be associated with losses in the colour, flavour and nutritional values of raw and processed foods (Serrano-Martínez, Fortea, del Amor, & Núñez-Delicado, 2008).

In the case of grapes, the enzymatic oxidation of phenolics leads not only to the browning of the flesh and loss of nutritional and organoleptic properties, but also to anthocyanin degradation (Yokotsuka & Singleton, 1997). These anthocyanins are directly degraded by PPO (Raynal & Moutounet, 1989) and by the quinones formed from the phenol substrates oxidized by PPO or POD (Yokotsuka & Singleton, 1997). Thus, the colour of this type of table grape is believed to be largely influenced by the composition of the anthocyanins and oxidizable phenols, as well as the degree of PPO and POD activity (Yokotsuka & Singleton, 1997).

The objective of this work was to extract and characterise PPO and POD from Crimson Seedless grape and to determine their kinetic parameters and thermal stability, in order to maximise the quality and minimise the economic and nutritional loss induced by these two oxidative enzymes, during the storage or processing of grape products such as purees or juices.

2. Materials and methods

2.1. Sampling and raw material

Crimson Seedless grapes were picked at maturity from a "parral" (horizontal shoot training) vineyard located in Murcia (South-East of Spain). Three replications of 10 vines each were selected and 1.5 kg random sample were picked from each replication. Samples were transported to the laboratory and frozen at −80 °C until they were used as PPO and POD source. Samples of frozen grapes (50 g) were picked at random to extract PPO and POD. All extracts were made in triplicate.

2.2. Reagents

Reagents were purchased from Sigma (Madrid, Spain) and used without purification. Triton X-114 was obtained from Fluka and was condensed three times as described by Bordier (1981) using 100 mM sodium phosphate buffers (pH 7.3). The detergent-rich phase of the third condensation had a concentration of 25% TX-114 (w/v).

The hydrogen peroxide solutions were freshly prepared every day, and their concentrations were calculated using $\varepsilon_{240} = 39.4$ M^{-1} cm^{-1} (Nelson & Kiesow, 1972).

2.3. PPO and POD extraction

Crimson Seedless grape PPO and POD were extracted using the method described by Núñez-Delicado, Sánchez-Ferrer et al. (2005a). All extractions were made in triplicate as explained below.

Grapes berries (50 g) were washed and homogenised for 1 min with 50 ml of 100 mM sodium phosphate buffer (pH 7.3) containing 10 mM ascorbic acid. The homogenate was filtered through eight layers of gauze and centrifuged at 4000 *g* for 15 min. The supernatant was discarded and the precipitate was extracted with 20 ml of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The mixture was subjected to temperature-induced phase partitioning and kept at 4 °C for 15 min and then warmed to 37 °C for 15 min. At this time, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins, anthocyanins and phenolic compounds. This turbid solution was centrifuged at 10,000*g* 10 min at 25 °C. After centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant, which was used as enzyme source, was stored at −20 °C.

2.4. Determination of proteins

The protein content was determined according to Bradfords dye binding method, using bovine serum albumin (BSA) as a standard (Bradford, 1976). Analyses were made in triplicate for each sample.

2.5. Enzyme activity

The PPO activity was followed spectrophotometrically at 400 nm in a Shimadzu model UV-1603 spectrophotometer (Kyoto, Japan) using TBC as a substrate ($\varepsilon_{400} = 1150$ M^{-1} cm^{-1}) (Núñez-Delicado, Sánchez-Ferrer et al., 2005a). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butyl-*o*-quinone per minute.

The standard reaction medium at 25 °C contained 2.6 μg/ml of partially purified PPO, 10 mM sodium acetate buffer (pH 3.0) and 2.5 mM TBC in a final volume of 1 mL.

The POD activity was followed spectrophotometrically in a Shimadzu model UV-1603 spectrophotometer at the absorption maximum of the ABTS radical cation, 414 nm ($\varepsilon_{414} = 31.1$ mM^{-1} cm^{-1}) (Rodríguez-López et al., 2000b). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of ABTS radical per minute.

The standard reaction medium at 25 °C contained 17.7 μg/ml of partially purified peroxidase, 10 mM sodium acetate buffer (pH 4.5), 2 mM ABTS, 2 mM H_2O_2 and 0.2 mM tropolone to discard any contribution of polyphenol oxidase at the progress of the reaction, in a final volume of 1 mL.

In the SDS standard assay, samples contained the above mixture and 2 mM SDS detergent in the cuvette.

Each sample was assayed in triplicate and the mean and standards deviation were plotted.

2.6. Thermal stability

The enzyme solutions (in Eppendorf tubes) were incubated in a circulating water bath Julabo Shake Temp SW 22 at different temperatures (60, 70, 75 and 80 °C) for different times (up to 40 min). After heating, samples were cooled in ice water and assayed immediately at 25 °C.

3. Results and discussion

The purple skin colour of Crimson Seedless table grape cultivars hinders the extraction of PPO and POD as a clear solution free of phenols and anthocyanins, since it interferes with the spectrophotometric determination of the enzymes. Therefore, PPO and POD were extracted using the Triton X-114 method (Sánchez-Ferrer, Bru, & García-Carmona, 1989a) which has been used for the purification of other purple skinned grapes PPOs (Núñez-Delicado,

Serrano-Mejías et al., 2005b). Other methods have been described in the literature such as the use of acetone powders, ammonium sulphate fractionation, salts, insoluble polymers and detergents (Núñez-Delicado et al., 2003), but, in our cases, the Triton X-114 method permitted phenolics and anthocyanins to be removed, yielding a PPO and POD in a clear solution. Indeed, the removal of phenols by TX-114 was sufficient to avoid browning of the enzyme solution, even after many cycles of freezing and thawing or after months of storage at −20 °C. This method has been used to separate hydrophobic proteins (Bordier, 1981) and remove phenolic compounds (Núñez-Delicado et al., 1996) and chlorophylls (Sánchez-Ferrer, Villalba, & García-Carmona, 1989b).

The pH is a determining factor in the expression of enzymatic activity. Figs. 1 and 2 show that Crimson Seedless PPO and POD were activated by acid shock. In the case of PPO, activation by acid shock (Cabanes et al., 2007) or basic shock (Chisari, Barbagallo, & Spagna, 2008) has been widely described. The pH scan for PPO (Fig. 1, open circles) revealed that the enzymatic activity is low from pH 7.5 to 5.5 but increases rapidly to reach a plateau of high activity from 4.5 to 3.0. This effect has previously been described for other vegetables PPOs, including persimmon (Núñez-Delicado et al., 2003), green bean (Jiménez & García-Carmona, 1996), peach (Cabanes et al., 2007) and, in other varieties of grape (Núñez-Delicado, Sánchez-Ferrer et al., 2005a; Núñez-Delicado, Serrano-Mejías et al., 2005b; Núñez-Delicado et al., 2007).

With respect to the pH scan for POD, the activity was studied using ABTS as H-donor. Fig. 2 (open squares) shows the pH profile for the oxidation of ABTS by POD, where it can be seen that the enzymatic activity increased as the pH decreased. Reports in the literature describe how the optimum pH of any peroxidase depends on the H-donor in the activity assay (Halpin, Pressey, Jen, & Mondy, 1989). In some papers, guaiacol has been used as the substrate (Chisari, Barbagallo, & Spagna, 2007; Chisari et al., 2008), while in other papers ABTS has been used (Duarte-Vázquez, García-Almendárez, Regalado, & Whitaker, 2000; Rodríguez-López et al., 2000a). Moreover, it has been described that the optimum pH depends of the POD source (Duarte-Vázquez et al., 2000; Serrano-Martínez et al., 2008). The POD assays described here were carried out at pH 4.5 in order to compare our results with those obtained in previous papers for POD from different sources (Rodríguez-López et al., 2000a; Serrano-Martínez et al., 2008).

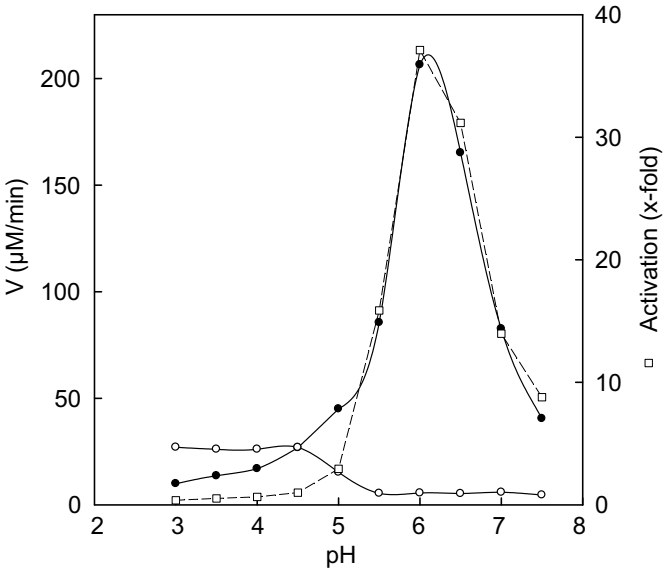

Fig. 1. Effect of pH on Crimson Seedless PPO activity in 10 mM sodium acetate buffer (pH 3.0–5.0) and 10 mM sodium phosphate buffer (pH 6.0–7.5), in the absence (○) or in the presence of 2 mM SDS (●). The reaction medium at 25 °C contained 2.5 mM TBC and 2.6 μg/ml enzyme (○) or 25 mM TBC, 2.6 μg/ml enzyme and 2 mM SDS (●). Activation degree at different pHs (□).

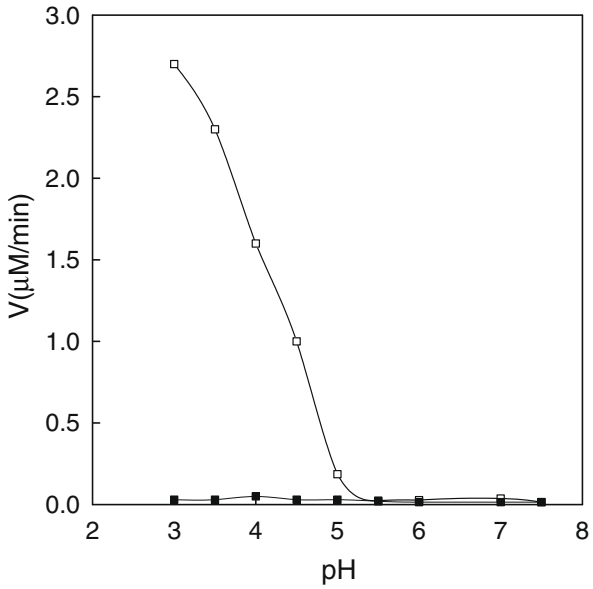

Fig. 2. Effect of pH on Crimson Seedless POD activity in 10 mM sodium acetate buffer (pH 3.0–5.0) and 10 mM sodium phosphate buffer (pH 6.0–7.5), in the absence (□) or in the presence of 2 mM SDS (■).The reaction medium at 25 °C contained 2 mM H_2O_2, 2 mM ABTS, 0.2 mM tropolone and 17.7 μg/ml enzyme.

The activating or inhibiting effect of SDS on different enzymes, including PPO and POD has been widely described. In the case of PPO, latent PPOs can be activated by SDS (Moore & Flurkey, 1990; Jiménez & García-Carmona, 1996; Saeidian, Keyhani, & Keyhani, 2007), while SDS has an inhibitory effect on POD (Nazari, Mahmudi, Shahrooz, Khodafarin, & Moosavi-Movahedi, 2005).

In order to study, the effect of SDS on the PPO and POD from Crimson Seedless grapes, their pH profiles were analysed in the presence of the detergent, many changes begin observed in both cases (Fig. 1, filled circles; Fig. 2, filled squares).

In the case of PPO (Fig. 1, open circles), SDS eliminated the acidic pH optimum observed on its absence and a new maximum appeared at pH 6.0 (Fig. 1, filled circles). This effect of SDS has previously been described for other latent PPOs extracted from different vegetables using TX-114 as extraction method and TBC as substrate (Bru, Sánchez-Ferrer, & García-Carmona, 1990; Cabanes et al., 2007; Núñez-Delicado, Sánchez-Ferrer et al., 2005a).

With regards to POD, the effect of 2 mM SDS was to inactivate the enzyme at all the studied pH values (Fig. 2, filled squares), an effect previously described for horseradish POD (Nazari et al., 2005).

In PPO, when the degree of the activation in the presence of 2 mM SDS at each pH was calculated, the greatest degree was obtained at pH 6.0 (40-fold) (Fig. 1, open squares). This activation was similar to that obtained for Dominga grape PPO (43.7-fold) (Núñez-Delicado, Serrano-Mejías et al., 2005b).

The effect of SDS depended on surfactant concentration in both cases, as shown in Fig 3. The optimum SDS concentration for activating PPO was 2 mM (Fig. 3, open squares). At SDS concentrations above 2 mM, an inhibitory effect was observed. These results contrast with those obtained for Monastrell grape in which the activation process was saturable, reaching its maximum activation at 3.5 mM SDS (Núñez-Delicado, Sánchez-Ferrer et al., 2005a). The activation of latent PPOs has been attributed to a reversible conformational change in the protein (Jiménez & García-Carmona, 1996; Laveda, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 2000).

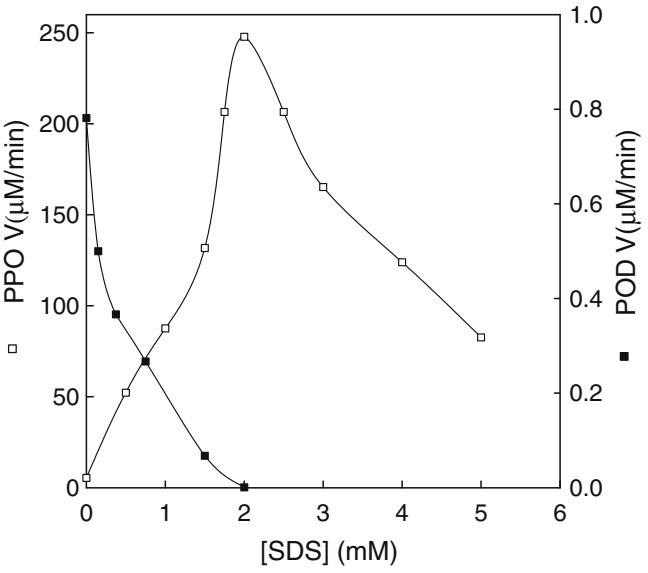

Fig. 3. Effect of SDS concentration on Crimson Seedless grape PPO and POD activity. In the case of PPO (□), the reaction medium at 25 °C contained 10 mM sodium phosphate buffer pH 6.0, 25 mM TBC, 2.6 μg/ml enzyme and increasing concentrations of SDS (0–5 mM). In the case of POD (■), the reaction medium at 25 °C contained 10 mM sodium acetate buffer pH 4.5, 2 mM H_2O_2, 2 mM ABTS, 0.2 mM tropolone, 17.7 μg/ml enzyme and increasing concentrations of SDS (0–2 mM).

SDS would induce a conformational change in the protein, leading to a pH profile shift (Moore & Flurkey, 1990; Selles-Marchat, Casado-Vela, & Brú-Martínez, 2007). It has been observed that some unsaturated fatty acids such as linolenic acid have a similar effect to SDS on PPO activity, causing inhibition at pH 4.5.

In the case of POD (Fig. 3, filled squares), the enzymatic activity decreased as SDS concentration increased, reaching total inactivation at 2 mM SDS. This effect has been previously described for horseradish POD (Nazari et al., 2005).

To further characterise Crimson Seedless grape PPO and POD, a detailed study of their kinetic parameters was carried out.

The study of the kinetic parameters, maximum rate (V_m) and Michaelis constant (K_m), of Crimson Seedless grape PPO was carried out with the enzyme activated by acid shocking at pH 3.0 and with the same enzyme activated by 2 mM SDS at pH 6.0, using TBC as substrate (Fig. 4A). The apparent kinetic parameters (V_m and K_m) were calculated by nonlinear regression to the Michaelis–Menten equation using the data obtained at pH 3.0 and pH 6.0 in the presence of 2 mM SDS. Fig. 4A shows variations in initial velocity versus substrate concentration in these conditions. The value obtained for each maximum velocity (V_m = 53.7 μM/min at pH 3.0 and V_m = 291.2 μM/min at pH 6.0 with 2 mM SDS) showed that the PPO activity was 5-fold higher for the enzyme activated by SDS (Fig. 4A, filled circles) than that activated by acid shocking (Fig. 4A, open circles). Moreover, the K_m value obtained with the enzyme activated by acid shocking (2.8 mM) was lower than that obtained with the enzyme activated by SDS (12.76 mM). The V_m/K_m ratio was 0.019 min^{-1} for the enzyme activated by acid shocking and 0.023 min^{-1} for the enzyme activated by SDS, indicating that Crimson Seedless PPO presents similar affinity for TBC in both cases.

At pH 3.0 and in the absence of SDS (Fig. 4A, open circles), the K_m value was in the same order as that obtained for other grape varieties, such as Monastrell grape (Núñez-Delicado, Sánchez-Ferrer et al., 2005a), Napoleon grape (3 mM; Núñez-Delicado et al., 2007) and Dominga grape (3 mM; Núñez-Delicado, Serrano-Mejías et al., 2005b).

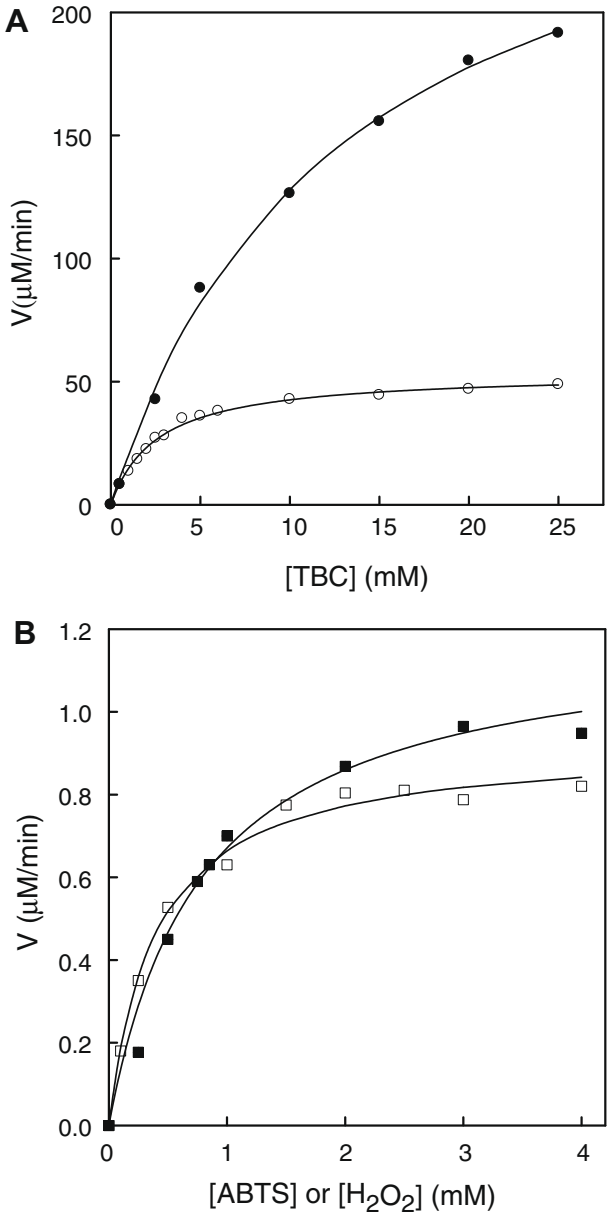

Fig. 4. (A) Effect of substrate concentration on Crimson Seedless PPO activity. The reaction medium at 25 °C contained 2.6 μg/ml enzyme and TBC concentrations ranging from 0 to 6 mM in 10 mM sodium acetate buffer (pH 3.0) (○), or 2.6 μg/ml enzyme, 2 mM SDS and TBC concentrations ranging from 0 to 25 mM in 10 mM sodium phosphate buffer (pH 6.0) (●). (B) Effect of substrates concentration on Crimson Seedless POD activity. The reaction medium contained 17.7 μg/ml enzyme, 0.2 mM tropolone, 2 mM H_2O_2 and ABTS concentrations ranging from 0 to 4 mM in 10 mM sodium acetate buffer pH 4.5 (■), or 17.7 μg/ml enzyme, 0.2 mM tropolone, 2 mM ABTS and H_2O_2 concentrations ranging from 0 to 4 mM in 10 mM sodium acetate buffer pH 4.5 (□).

In the presence of SDS (Fig. 4A, filled circles), the K_m obtained was approximately double that obtained for Napoleon (8 mM; Núñez-Delicado et al., 2007) and Dominga (8 mM; Núñez-Delicado, Serrano-Mejías et al., 2005b) grape PPO.

In the case of Crimson Seedless grape POD, when the ABTS concentration was increased, the steady-state rate also increased up to saturation at 2 mM (Fig. 4B, filled squares). The K_m value for ABTS was found to be 0.79 mM and the V_m value was 1.20 μM/min. In addition, when the H_2O_2 concentration was increased, at a fixed, saturating concentration of ABTS, the activity increased to reach saturation at 1.5 mM, obtaining a K_m value of 0.4 mM and a V_m

value of 0.93 μM/min (Fig. 4B, open squares). These results are in concordance with those obtained for pepper POD (Serrano-Martínez et al., 2008), but contrast with those described for horseradish, asparagus and turnip peroxidases (Duarte-Vázquez et al., 2000; Hiner, Hernández-Ruiz, Arnao, García-Cánovas, & Acosta, 1996; Rodrigo, Rodrigo, Alvarruiz, & Frigola, 1996), in which an inhibition by substrate concentration was described for H_2O_2.

The K_m value obtained for ABTS (0.79 mM) was twice that described for turnip (0.47 mM; Duarte-Vázquez et al., 2000) or pepper POD (0.495 mM; Serrano-Martínez et al., 2008), and four times higher than that described for Brussels sprouts POD (0.2 mM; Regalado, Pérez-Arvizu, García-Almendarez, & Whitaker, 1999). However, it was about five times lower than that described for horseradish POD (4 mM; Hiner et al., 1996). The K_m value obtained for H_2O_2 (0.4 mM) was lower than those obtained for other peroxidases such us pepper (1.32 mM; Serrano-Martínez et al., 2008) or turnip POD (0.850 mM, Duarte-Vázquez et al., 2000), but was double that described for melon POD (0.23 mM; Rodríguez-López et al., 2000a).

To complete the study of Crimson Seedless grape PPO and POD, the thermal stability of both enzymes was studied. The semi-log plots of the residual activity of PPO (Fig. 5A) and POD (Fig. 5B) versus heating time were linear at all temperatures studied, which is consistent with inactivation by means of a simple first-order process for both. The fact that the lines all extrapolate back to a common point indicates that the inactivation of the sole isoenzyme is being measured in each case (PPO, Fig. 5A and POD, Fig. 5B). This result is in accordance with those obtained for other PODs from pepper (Serrano-Martínez et al., 2008), potato or carrot (Anthon & Barrett, 2002), but contrasts with those obtained for potato and carrot PPO (Anthon & Barrett, 2002). From the slopes of these lines, the inactivation rate constants (k) were calculated by linear regression according to the equation (Anthon & Barrett, 2002):

$$\log\left(\frac{A}{A_0}\right) = -\left(\frac{k}{2.303}\right) \times t$$

where A_0 is the initial enzyme activity and A is the activity after heating for time t. The k values obtained were plotted in an Arrhenius plot (Fig. 6). The Arrhenius plot for PPO (Fig. 6, filled circles) and POD (Fig. 6, open circles) showed different upward curvatures. At temperatures >70 °C (i.e., at $1/T < 0.00292$) the plots can be approximated by straight lines in both cases. From the slopes of these lines, activation energies (E_a) of 295.5 kJ/mol for PPO and 271.9 kJ/mol for POD were calculated by the equation (Anthon & Barrett, 2002)

$$\ln(k) = -\frac{E_a}{RT^{+c}}$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in K. This curvature has previously been described for potato and carrot PODs (Anthon & Barrett, 2002) and green bean and pea seed lipoxygenases (Busto et al., 1999; Indrawati, Van Loey, Ludikhuyze, & Hendrickx, 1999). The simplest explanation for such curvature is that inactivation occurs through more than one mechanism, each with its own temperature dependence. The observed overall temperature dependence is simply the sum of the individual processes. At temperatures >70 °C (i.e., at $1/T < 0.00292$), inactivation is the result of a process, such as protein unfolding, with a high activation energy. The high E_a necessarily means that the rate of this process is strongly-temperature dependent, and so at lower temperatures this rate becomes insignificant. At these lower temperatures, therefore, the observed rate reflects some other process with lower activation energy, such as the loss of some functional group or the dissociation of the heme group in the case of POD. Both protein denaturation and loss of some functional group were shown to be mechanisms by which Crimson Seedless PPO and POD were inactivated.

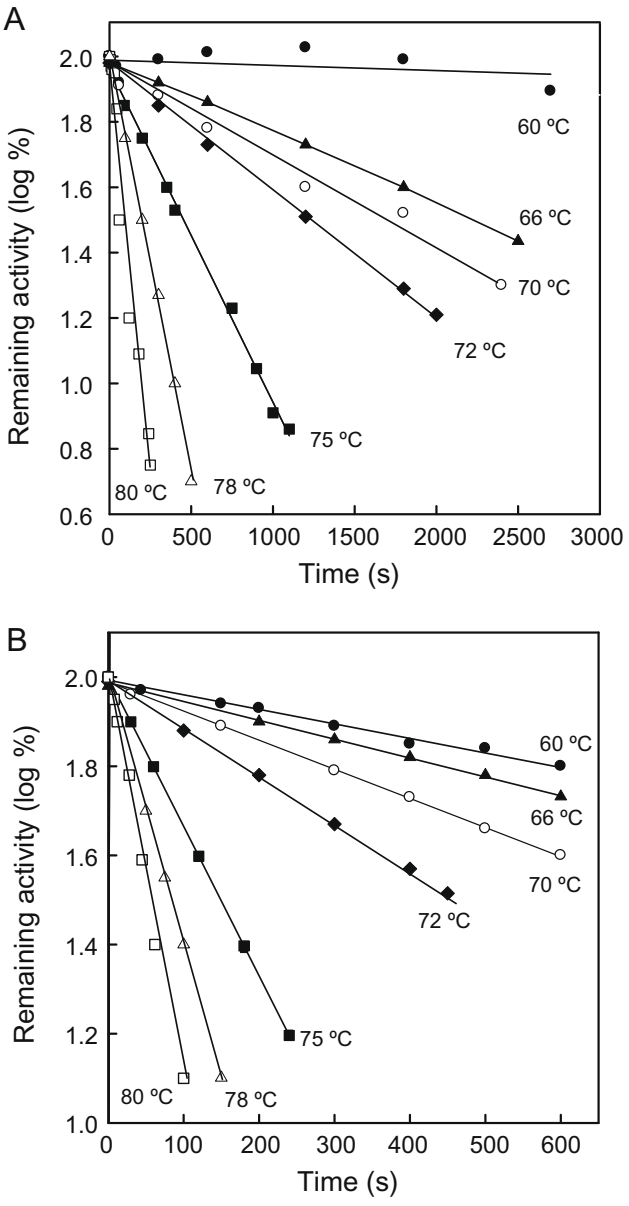

Fig. 5. Heat inactivation of Crimson Seedless grape PPO and POD. (A) Remaining PPO activity versus heating time. The reaction medium contained 10 mM sodium acetate buffer (pH 3.0), 2.5 mM TBC and 2.6 μg/ml. (B) Remaining POD activity versus heating time. The reaction medium contained 10 mM sodium acetate buffer (pH 4.5), 2 mM H_2O_2, 2 mM ABTS, 0.2 mM tropolone and 17.7 μg/ml.

The E_a value obtained for Crimson Seedless grape PPO (295.5 kJ/mol) was similar to that obtained for POD (271.9 kJ/mol), using the same range of inactivation temperatures (60–80 °C). The E_a value obtained for PPO (295.5 kJ/mol) was about two times higher than that obtained for "Elsanta" and "Madame Moutot" strawberry PPO (Chisari et al., 2007) or "Amarillo" melon PPO (Chisari et al., 2008) and ten times than that obtained for "Charantais" melon (Chisari et al., 2008). The range of temperatures required for the inactivation of Crimson Seedless grape PPO was also higher than those observed for strawberry (Chisari et al., 2007) or melon PPO (Chisari et al., 2008), with significant inactivation at temperatures >70 °C. These results indicated that Crimson Seedless grape PPO is more thermostable than strawberry or melon PPO.

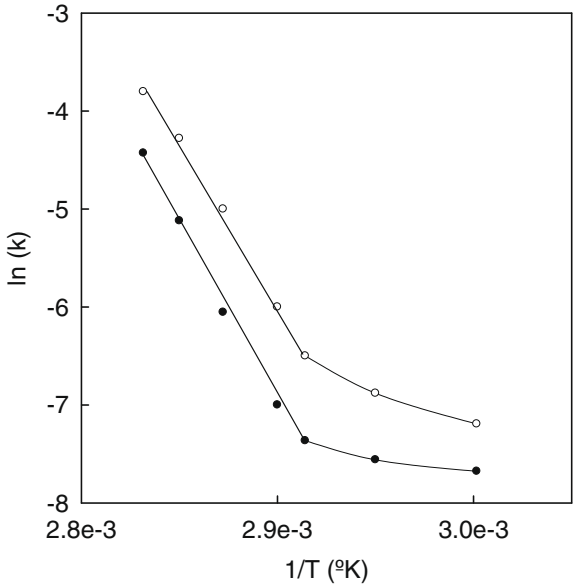

Fig. 6. Arrhenius plot of inactivation rates from PPO (●) and POD (○).

In the case of POD, the E_a value obtained (271.9 kJ/mol) was about half that obtained for potato or carrot POD (Anthon & Barrett, 2002), but was about double that obtained for "Amarillo" melon (Chisari et al., 2008) or pepper POD (Serrano-Martínez et al., 2008), and three times higher than those obtained for "Charantais" melon (Chisari et al., 2008) or "Elsanta" and "Madame Moutot" strawberry POD (Chisari et al., 2007). The range of temperatures required for the inactivation of Crimson Seedless grape POD was similar to that obtained for potato and carrot PODs (Anthon & Barrett, 2002), but higher than that for strawberry (Chisari et al., 2007), melon (Chisari et al., 2008), and pepper PODs (Serrano-Martínez et al., 2008), with significant inactivation at temperatures >70 °C. These results indicated that Crimson Seedless grape POD is more thermostable than strawberry, melon or pepper POD, but it is less thermostable than potato and carrot POD.

In some cases, inactivation is given as the *D* value, the time required to reduce the enzyme activity to 10% of its original value. The temperatures required for a *D* value of 5 min were very similar for both enzymes studied. Crimson Seedless grape PPO and POD (78 °C and 75 °C, respectively). In the case of PPO, the *D* value of 5 min (78 °C) was similar to that described for melon and strawberry PPO (Chisari et al., 2007). In the case of POD the *D* value of 5 min (75 °C) was much higher than that obtained for pepper POD (44.5 °C, Serrano-Martínez et al., 2008), lower than that obtained for potato and carrot POD (80 °C; Anthon & Barrett, 2002) and similar to that described for melon and strawberry POD.

In conclusion, a detailed kinetic study of Crimson Seedless grape PPO and POD isolated using TX-114 is presented for the first time. PPO was presented in a latent state, and could be activated by acid schoking and the anionic detergent SDS. However, this anionic detergent inactivated POD. The kinetic study of the thermal stability of both enzymes showed that they are very thermostable. This high thermostability may be taking into account when thermal treatments were used to obtain processed products derived from grape. Moreover, POD can be used as an index of the adequacy of fruit and vegetables blanching.

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