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Kinetic characterisation and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase

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Abstract

Peroxidase (POD) from red sweet pepper cultured under an integrated system was partially purified, using a combination of phase partitioning with Triton X-114 and ammonium sulphate fractionation between 30 and 80%. The enzyme presented a single band in PAGE only when 4-MN was used as substrate. Optimum activity using ABTS as the H-donor was obtained at pH 4.5 and the apparent kinetic parameters V_m and K_M were calculated for both ABTS and H_2O_2, showing a K_M value in the same order in both cases (0.495 and 1.32 mM, respectively). The effect of several reducing agents was studied, ascorbic acid being the most active. The study of thermal inactivation showed a first-order inactivation kinetic, and the Arrhenius plot yielded a straight line with a slope equivalent to an activation energy of 151 kJ/mol. Significant inactivation occurred at temperature >40 °C and the D value for 5 min was 44.5 °C.

Keywords: Peroxidase; Pepper; Triton X-114; Thermostability

1. Introduction

Peroxidase (POD; EC 1.11.1.7) is an oxidoreductase that catalyses a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms (Adams, 1978; Rodrigo, Rodrigo, Alvarruiz, & Frigola, 1996; Whitaker, 1994). It is involved in enzymatic browning since diphenols may function as reducing substrates in this reaction (Robinson, 1991a). The involvement of POD in browning is limited by the availability of electron acceptor compounds such us superoxide radicals, hydrogen peroxide and lipid peroxides.

The enzyme is reported to exist in both soluble and membrane-bound forms (Robinson, 1991a) and is involved in many plant functions such as hormone regulation (Gaspar, 1986), defence mechanisms, indoleacetic acid degradation during maturation and senescence of fruits and vegetables and lignin biosynthesis (Thongsook & Barrett, 2005). Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants (Estrada, Bernal, Díaz, Pomar, & Merino, 2000).

Besides being an important factor in the physiology of plants, this enzyme is of great interest in food technology because of its influence on the quality of raw and processed fruits and vegetables (Morales, Pedreño, Muñoz, Ros Barceló, & Calderón, 1993; Prestamo & Manzano, 1993; Robinson, 1991b). In the presence of peroxide, POD produces phytotoxic free radicals which react with a wide range of organic compounds (ascorbic acid, carotenoids and fatty acids), leading to losses in the colour, flavour and nutritional value of raw and processed foods (Bruemmer, Roe, & Bowen, 1976; Kampis, Bartuczkovacs, Hoschke, & Aosvigyazo, 1984; Nebesky, Esselen, Kaplan, & Felleres, 1950; Robinson, 1987).

POD is also of concern to food processors because of its high thermostability. So, it is commonly used as an index of the adequacy of fruit and vegetable blanching due to

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its high concentration in plant tissues, its high thermal stability and its ease of assay (Anthon & Barrett, 2002; Lu & Whitaker, 1974). Heat treatment of vegetables had also led to the loss of desirable characteristics such as colour, texture, flavour and nutrients. For this reason, it is desirable to keep the heat treatment to a minimum yet still make it sufficient to inactivate completely the deleterious enzymes. The high thermal stability of POD can be seen as either an advantage or a problem. On the one hand, it provides a natural margin of safety in that, if POD is inactivated, it is a reasonable assumption that other quality-related enzymes will have been inactivated also. On the other hand, the reliance on POD as an indicator may lead to an excessive heat treatment of the product and cause other quality problems (Anthon & Barrett, 2002).

Peppers are popular vegetables because of the combination of colour, taste and nutritional value. Fresh pepper is one of the vegetables with the highest content of vitamin C (Vanderslice, Higgs, Hayes, & Block, 1990) and is a good source of provitamin A carotenoids. Moreover, the red pepper fruit (*Capsicum annuum* L.) has been used for many years as a source of pigments to add to or change the colour of foodstuffs, making them more attractive and acceptable for the consumer.

The aim of this work was to extract peroxidase from red pepper growing under an integrated system and characterise it by determination of kinetic parameters, optimum conditions of pH and temperature, thermal stability and inhibitory effects of reducing agents, using Triton X-114 (TX-114) to remove the carotenoid pigments from the extract.

2. Materials and methods

2.1. Plant material and growth conditions

Sweet pepper plants (*C. annuum* L.) cv. Almuden were transplanted from a commercial nursery on 14 December 2005. Plants were grown in a plastic greenhouse. The irrigation schedule was applied according to the US Weather Bureau Class A evaporation pan, which was placed inside the greenhouse. The greenhouse had automated control of relative humidity by a fog system. Sweet pepper plants were grown under integrated cultivation management. This farming method does make use of chemical fertilisers and plant protection products but incorporates as well all biological mechanisms to improve plant health and crop yield. Integrated farming methods are based on the main philosophy of low-input farming, reducing significantly nitrogen fertilisers and chemical pesticides whilst growth (del Amor, 2006) and yield (del Amor, 2007) are not affected in sweet pepper plants compared with conventional farming. Manure (4 kg m^{-2}) was applied pre-planting, combined with a reduced chemical fertiliser dosage through the crop cycle. Thus, we applied 16.4 mL m^{-2} of 75% phosphoric acid, 43.5 g m^{-2} of calcium nitrate, 58.7 g m^{-2} of potassium nitrate and 31.2 g m^{-2} of magnesium sulphate.

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 Brodier (1981), using 100 mM sodium phosphate buffer (pH 7.3). The detergent-rich phase of the third condensation had a TX-114 concentration of 25% (w/v).

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

2.3. Partial purification of pepper peroxidase

Pepper peroxidase was extracted and partially purified using the method described by our group in 2003, but with some modifications (Núñez-Delicado, Sojo, García-Carmona, & Sánchez-Ferrer, 2003).

Fresh peppers were washed and the seeds and peduncle were removed. A 50-g sample was homogenised with 100 mL of sodium phosphate buffer (pH 7.3) for 5 min in an Ultraturrax. The homogenate was filtered through four layers of cheesecloth. This filtrate was subjected to temperature-induced phase partitioning by adding TX-114 at 4 °C so that the final detergent concentration was 4% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 35 °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 and phenolic compounds. This turbid solution was centrifuged at 10,000*g* for 15 min, at 25 °C. After discarding the pellet and detergent-rich phase, the clear, detergent-poor supernatant, which contained the soluble pepper POD, was brought to 30% saturation with $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After one hour, the solution was centrifuged at 60,000*g*, for 30 min at 4 °C, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clear supernatant to give 80% saturation and the mixture was stirred for 1 h at 4 °C. The precipitate obtained between 30% and 80% was collected by centrifugation at the same rotor speed and dissolved in a minimum volume of 100 mM sodium phosphate buffer, pH 7.3. The salt content was removed by dialysis and the enzyme stored at −20 °C.

2.4. Enzymatic activity

The peroxidase 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., 2000). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of ABTSradical per minute.

The standard reaction medium, at 25 °C, contained 1 ng/mL of partially purified peroxidase, 50 mM sodium citrate buffer (pH 4.5), 1 mM ABTS, 6 mM H_2O_2 and 0.2 mM tropolone, in a final volume of 1 mL.

3. Electrophoresis

PAGE was carried out as described by Angleton and Flurkey (1984). Samples were mixed with glycerol, bromophenol blue before being applied to 8% polyacrylamide gels. Electrophoresis was carried out for 1 h at 25 °C in a Mini protein cell (Bio-Rad). The gels were stained for peroxidase activity in 100 mL of 50 mM sodium acetate buffer (pH 5.0) containing 0.45 mM H_2O_2 and 1 mM 4-methoxy-α-naftol (4MN) (Bru, Sellés, Casado-Vela, Belchí-Navarro, & Pedreño, 2006).

4. Determination of proteins

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

4.1. Total phenolics

Total phenolic compounds were determined in 80% ethanol using the Folin–Denis method (Kidron, Harel, & Mayer, 1978). Analyses were done in triplicate for each sample, using chlorogenic acid for the quantification.

4.2. Thermal stability

The enzyme solutions in Eppendorf tubes were incubated in a circulating water bath at different temperatures (30, 40, 50 and 60 °C) for different times, up to 10 min. After heating, samples were cooled in ice water and assayed immediately at 25 °C.

5. Results and discussion

Enzyme purification in plant extracts is a difficult process because of the presence of a large variety of secondary products that can bind tightly to the enzymes and change their characteristics (Loomis, 1974). To overcome this problem, different methods have been developed, such as the use of acetone powders, ammonium sulphate fractionation, salts, insoluble polymers and detergents. Among the latter, TX-114 shows the special feature of forming clear solutions in buffers at 4 °C, whereas it separates into two-phases at 20 °C due to the formation of large micellar aggregates. This has been used to separate hydrophobic proteins (Brodier, 1981) and to remove phenolic compounds (Núñez-Delicado, Bru, Sánchez-Ferrer, & García-Carmona, 1996; Núñez-Delicado et al., 2003) and chlorophylls (Sánchez-Ferrer, Bru, & García-Carmona, 1989).

In the present paper, red pepper fruit POD was partially purified using a combination of phase partitioning in TX-114 and ammonium sulphate fractionation, in order to avoid changes in the enzyme characteristics due to the binding of secondary products and to remove the carotenoid pigments from the extract, to obtain a clear solution as the enzyme source.

Due to the presence of large quantities of vitamin C in pepper fruit, it was not necessary to add TX-114 in the first step of the extraction method, to avoid the enzymatic browning of the extract. So, after homogenisation and centrifugation of pepper fruit in the presence of buffer, a red solution was obtained, termed crude extract in the purification table (Table 1). To remove the carotenoid pigments responsible for the red colour of the extract, 4% (w/v) TX-114 was added and the solution phase-partitioned by increasing the temperature to 37 °C and holding for 15 min. This two-phase step involved a loss of proteins (41%), a slight loss of POD activity (12%) and a 50% reduction in the level of phenols (Table 1) and gave a transparent supernatant without red colour. In the following step, the ammonium sulphate fractionation gave a 2.3-fold purification with 66% recovery (Table 1).

The reduction of phenolic compounds to only 18% of the original content is similar to that described in the TX-114 purification of potato tuber, mushroom and persimmon polyphenol oxidase (Núñez-Delicado et al., 1996; Núñez-Delicado et al., 2003; Sánchez-Ferrer, Laveda, & García-Carmona, 1993). 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. Moreover, the use of TX-114 gave a clear enzymatic extract, without red pigments that could interfere in the enzymatic measurement.

The partially purified red pepper POD appeared as a single band in PAGE (Fig. 1, inset) when 4-methoxy-α-naftol (4MN) was used as substrate. This result contrasts with those described in the literature, since multiple isoenzymatic bands have been described for POD extracted from different plant sources (Duarte-Vázquez, García-Almendárez, Regalado, & Whitaker, 2000). This may be because the extraction method used in the present paper permitted the extraction of a unique isoenzymatic form and the

Table 1
Partial purification of red pepper POD

	Vol (mL)	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)	Phenolic compounds (mg/mL)
Crude extract	130	76.2	298.7	3.9	1	100	0.6
Supernatant 4% TX-114	113	44.9	262.5	5.8	1.5	88	0.3
30–80 % $(NH_4)SO_4$	6	21.9	195.7	8.9	2.3	66	0.1

[a] Assayed with ABTS as H-donor.

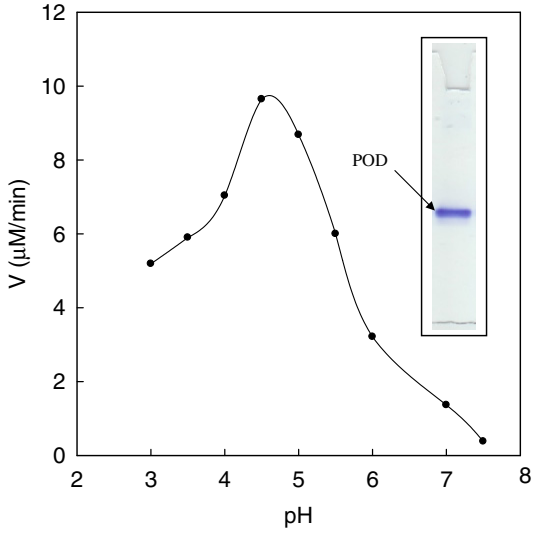

Fig. 1. Effect of pH on partially purified red pepper POD activity in 50 mM sodium citrate buffer (pH 3.0–5.5) and 50 mM sodium phosphate buffer (pH 6.0–7.5). The reaction medium at 25 °C contained 1 mM ABTS, 6 mM H_2O_2, 0.2 mM tropolone and 1 ng/mL of partially purified POD. (Inset) SDS-PAGE (8% gel) of red pepper POD stained in 50 mM sodium citrate buffer (pH 5.0) containing 0.45 mM H_2O_2 and 1 mM 4-metoxi-α-naftol (4MN).

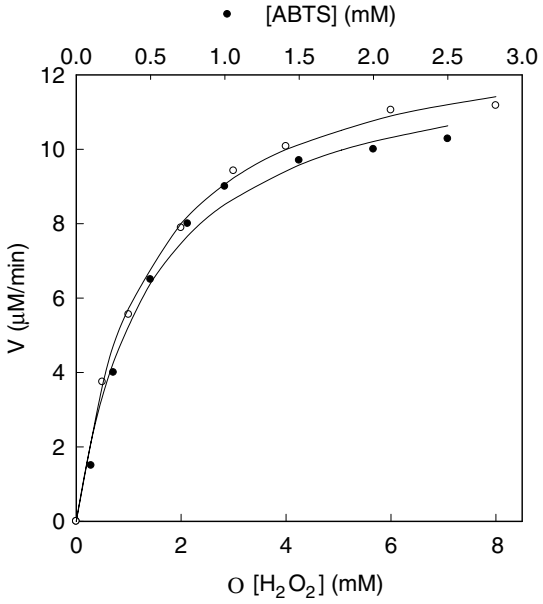

Fig. 2. Effect of ABTS and H_2O_2 concentration on partially purified red pepper POD activity. The reaction medium contained 1 ng/mL of partially purified POD, 50 mM sodium citrate buffer pH 4.5, 0.2 mM tropolone and: 6 mM H_2O_2 and ABTS concentrations ranging from 0 to 2.5 mM (●), or 1 mM ABTS and H_2O_2 concentrations ranging from 0 to 8 mM (○).

removal of phenols avoided the modification of POD isoenzymes.

The enzymatic activity of partially purified red pepper POD was measured using ABTS as the H-donor compound and H_2O_2 as the acceptor, in the presence of tropolone to discard any contribution of polyphenol oxidase to the progress of the reaction. Fig. 1 shows the pH profile for the oxidation of ABTS, indicating the optimum pH to be 4.5. This acidic pH indicates that the POD isoenzymatic form extracted from red pepper with our method was an acidic POD. This is in accordance with results obtained for turnip POD using ABTS as H-donor (Agostini, Medina, Milrad de Forchetti, & Tigier, 1997; Duarte-Vázquez et al., 2000). It is known that the optimum pH for any peroxidase depends on the H-donor in the activity assay (Halpin, Pressey, Jen, & Mondy, 1989).

The apparent kinetic parameters (V_m and K_M) for ABTS and H_2O_2 were calculated. The K_M value for ABTS was 0.495 mM (Fig. 2, filled circles). In addition, when the H_2O_2 concentration was increased, at a fixed, saturating concentration of ABTS, the activity increased up to saturation at 6 mM (Fig. 2, open circles), obtaining a K_M value of 1.32 mM. These results are in contrast with those described for horseradish, asparagus and turnip peroxidases (Arnao, Acosta, del Río, Varón, & García-Cánovas, 1990; Duarte-Vázquez et al., 2000; Hiner, Hernández-Ruiz, Arnao, García-Cánovas, & Acosta, 1996; Rodrigo et al., 1996), in which an inhibition by substrate concentration was described for H_2O_2. The K_M value obtained for ABTS was similar to that described for the C3 fraction of turnip peroxidase (0.47 mM; Duarte-Vázquez et al., 2000), whereas it was about ten times lower than that described for acidic horseradish peroxidase (4 mM; Hiner et al., 1996) and two times higher than that described for Brussels sprouts peroxidase (0.2 mM; Regalado, Pérez-Arvizu, García-Almendárez, & Whitaker, 1999). The K_M value obtained for H_2O_2 was much higher than the values obtained for other peroxidases (Duarte-Vázquez et al., 2000; Regalado et al., 1999). It is important to know the apparent K_M values for ABTS and H_2O_2 because of the use of peroxidase as a reporter enzyme for immunoassays.

ABTS oxidation was found to be dependent on the amount of extract used in the assay and was linear up to 2 ng/mL of protein. No activity was observed when boiled extract (10 min at 100 °C) was used (data not shown).

To further characterise the partially purified enzyme, a study of its inhibition by reducing agents was carried out. Fig. 3 shows the effect of various reducing agents (ascorbic acid, L-cysteine and metabisulphite) on the partially purified red pepper POD, using ABTS as substrate. Ascorbic acid, which is present at a high level in pepper, appeared to be the most effective inhibitor (Fig. 3), acting as an antioxidant rather than as an enzyme inhibitor because it reduces the ABTS radical to its original form. The inhibition produced by the thiol compounds studied (L-cysteine and metabisulphite) was lower, and may be due to an addition reaction taking place with the ABTS radical to form stable products and/or a binding to the active centre of the enzyme as in the case of metabisulphite with polyphenol oxidase (Fig. 3, inset) (Ikediobi & Obasuyi, 1982; Valero, Varón, & García-Carmona, 1992).

To complete the study of partially purified red pepper POD, its thermal stability was studied. The semi-log plots of the residual activity versus heating time were linear at

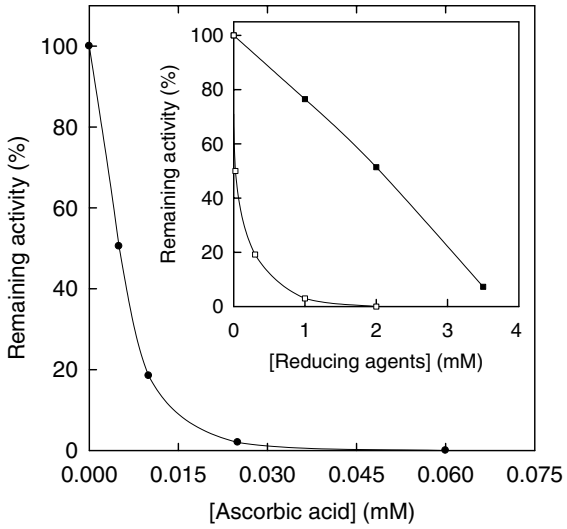

Fig. 3. Inhibitory effect of ascorbic acid on partially purified red pepper POD using ABTS as substrate. The reaction medium at 25 °C contained 50 mM sodium citrate buffer pH 4.5, 1 mM ABTS, 6 mM H_2O_2, 0.2 mM tropolone, 1 ng/mL of partially purified POD and ascorbic acid concentrations ranging from 0 to 0.06 mM. (Inset) Effect of L-cysteine (■) and metabisulphite (□), ranging from 0 to 3.5 mM.

all temperatures studied (Fig. 4), consistent with inactivation occurring by a simple first-order process. The fact that the lines all extrapolate back to a common point indicates that the inactivation of the unique isoenzyme is being measured in each case. From the slopes of these lines, inactivation rate constants, were calculated by linear regression and the rate constants (k) were calculated according to the equation (Anthon & Barrett, 2002):

$$\log(A/A_0) = -(k/2.303)t,$$

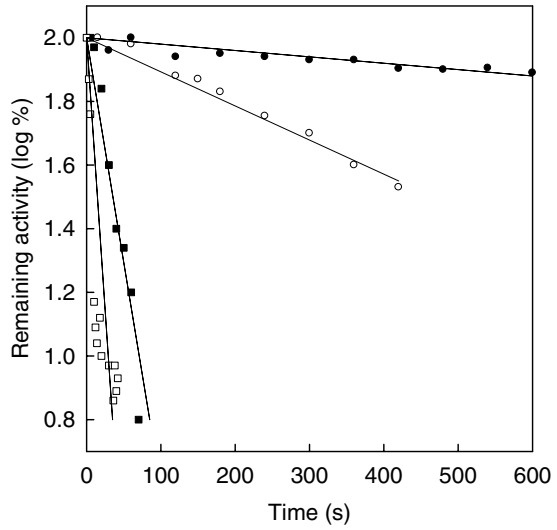

Fig. 4. Heat inactivation of partially purified red pepper POD. Remaining activity versus heating time at 30 °C (●), 40 °C (○), 50 °C (■) and 60 °C (□). The reaction medium contained 50 mM sodium citrate buffer pH 4.5, 1 mM ABTS, 6 mM H_2O_2, 0.2 mM tropolone and 1 ng/mL of partially purified POD.

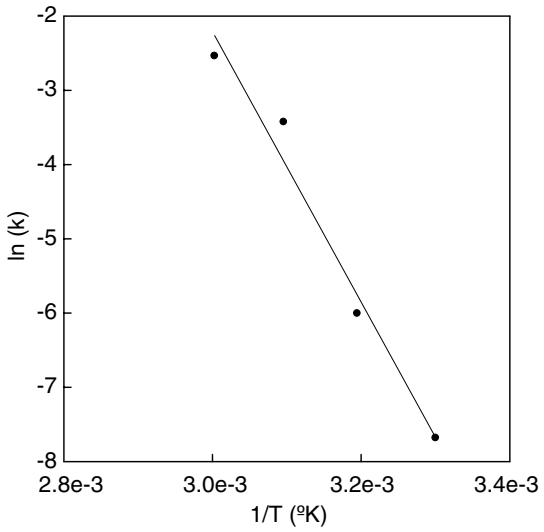

Fig. 5. Arrhenius plot of the inactivation rates in Fig. 4.

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. 5), showing a simple linear fit. The activation energy (E_a) was calculated from the slope of the Arrhenius plot, by the equation (Anthon & Barrett, 2002):

$$\ln(k) = 52.4 - \left(\frac{E_a}{R} * \frac{1}{T}\right),$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in K. The value obtained for E_a was 151 kJ/mol. Although this activation energy is lower than that obtained for carrot and potato POD (Anthon & Barrett, 2002) and slightly higher than that obtained for strawberry POD (Chisari, Barbagallo, & Spagna, 2007), the range of temperatures required for the inactivation of red pepper POD was lower, with significant inactivation at temperatures >40 °C. 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 temperature required for a D value of 5 min was 44.5 °C, a lower value than for PODs from potato and carrot (83.2 and 80.3 °C, respectively) (Anthon & Barrett, 2002), indicating that red pepper POD is less thermostable than those PODs. Moreover, the time required to halve the activity at 60 °C was 6 s. This is much less than for strawberry POD (4 min) (Chisari et al., 2007), indicating again that red pepper POD is less thermostable than strawberry POD.

In conclusion, the results of this paper show, for the first time, a detailed kinetic study of red pepper POD, which presents a single band in electrophoresis when isolated using TX-114. The thermal inactivation study of this POD shows that it is a very thermolabile enzyme in comparison with PODs extracted from other plant sources.

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