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Kinetic characterisation and thermal inactivation study of red alga (*Mastocarpus stellatus*) peroxidase

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ABSTRACT

Peroxidase (POD) was extracted from red alga (*Mastocarpus stellatus*) using Triton X-114 and characterised by UV-spectrophotometry. Optimum activity using 2,2́-azinobis(3-ethylbenzothiazolinesulphonic acid) (ABTS) as the H-donor was obtained at pH 5.0. In the presence of the anionic detergent, sodium dodecyl sulphate (SDS), however, POD was inactivated at all the pH values studied and totally inactivated at 1 mM SDS. When the enzyme was kinetically characterised, the K_M and V_m values for ABTS were found to be 13 mM and 40 μM/min, respectively. In addition, when the H_2O_2 concentration was increased, at a fixed concentration of ABTS, the activity was inhibited at the highest H_2O_2 concentrations. In a study of the effect of several reducing agents, L-cysteine was found to be the most active. A thermal inactivation study showed a first-order inactivation kinetic, and the Arrhenius plot yielded a straight line with a slope equivalent to an activation energy of 121.6 kJ/mol. Significant inactivation occurred at temperatures of >35 °C, with >90% of the relative activity being lost after only 5 min of incubation at 48.4 °C.

1. Introduction

In 2003, it was estimated that approximately 1 million tonnes of wet seaweed were harvested in 35 countries as a source of food; agar, alginate and carrageenan; fertilizer; fuel; and for use in the cosmetic field (McHugh, 2003, chap. 8). However, the most common use of seaweed is as a dietary supplement. Such use has a long tradition, where seaweed has been considered an important dietary component since, at least, the fourth century in Japan and the sixth century in China (McHugh, 2003, chap. 8). Recently, these and other countries, such as the Republic of Korea, the United States of America, South America, Ireland, Iceland, Canada and France have significantly increased the consumption, production and marketing of seaweeds (McHugh, 2003, chap. 8). As demand has increased, natural stocks have been unable to meet market requirements, and now more than 90% of seaweeds used commercially are cultivated (McHugh, 2003, chap. 8). Seaweeds are a valuable food source as they contain protein, lipids, vitamins and minerals. However, they are not always destined for human consumption, and whole plants, as well as seaweed mixes, have been used in animal nutrition and as fish feed (McHugh, 2003, chap. 8). Very few of the world's available seaweed species are used commercially, either because they cannot be harvested or cultivated on a commercially viable scale, or because their composition makes them unsuitable.

The underexploited Portuguese seaweed *Mastocarpus stellatus* is a known source of κ/ι carrageenans, a generic name given to a family of sulphated polysaccharides isolated from red seaweeds. These water-soluble linear biopolymers are increasingly used as natural thickeners, stabilizers or gelling agents in applications ranging from the food (mainly dairy products) to the pharmaceutical industry (Hilliou, Larotonda, Sereno, & Gonçalves, 2006).

Recently, the red alga *M. stellatus* has been deemed suitable for use as a biosorbent for cadmium removal from solutions because of its low cost (Herrero et al., 2008). In addition, marine macroalgae are considered as a promising source for new antimicrobial agents. Indeed, recent studies have shown that many macroalgae, e.g. *M. stellatus*, produce metabolites with antibacterial activities (Bazes et al., 2006; Dubber & Harder, 2008).

Peroxidases (POD; EC 1.11.1.7) are haem proteins that contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. These are a group of oxidoreductases that catalyse the reduction of peroxides, such as hydrogen peroxide coupled to the oxidation of a variety of organic and inorganic compounds. They are ubiquitous in nature and have diverse physiological

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functions, including the removal of H_2O_2, the biosynthesis and degradation of lignin in cell walls, auxin metabolism and defensive responses to wounding and defense against pathogens or insects (García-Lara, Arnason, Díaz-Pontones, Gonzalez, & Bergvinson, 2007). Peroxidases show wide substrate specificity, a characteristic that makes them useful in a number of industrial, analytical and biomedical applications. Peroxidases are used commercially as catalysts for phenolic resin synthesis and as components of medical diagnosis kits (Chung, Kim, Bernhardt, & Pyun, 2005). They are also used in the treatment of waste water containing phenolic compounds and aromatic amines, the removal of peroxide from materials such as foodstuffs and industrial wastes, as a labelling enzyme in immunochemistry and the characterisation of disease status in experimental pathology, as reagents for organic synthesis and bio-transformation, as well as in coupled enzyme assays, chemiluminescent assays and immuno assays (Krieg & Halbhuber, 2003).

The involvement of POD in browning processes has been reported by many researchers. However, the POD activity is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide, and lipid peroxides. POD is involved in these processes, because diphenols may function as reducing substrates in this reaction (Robinson, 1991).

The browning damage caused in the tissues of fruits and vegetables during postharvest handling and processing is one of the main causes of quality loss. Peroxidase, which is responsible for a multitude of quality and flavour alterations in fruits and vegetables (Vámos-Vigyázó, 1981), may be associated with losses in colour, flavour and the nutritional values of raw and processed foods (Serrano-Martínez, Fortea, del Amor, & Núñez-Delicado, 2008).

To prevent undesirable reactions, heat treatment, which inactivates the enzyme, is usually used for the preservation of many foods. However, the application of heat treatment is limited by alterations in sensory characteristics and the loss of nutrients which may be caused by this process (Bomber, Dietrich, Hudson, Hamilton, & Farkas, 1975). Consumer demand for fresh-like food products with minimal degradation of nutritional and organoleptic properties has stimulated research into new treatments and new products in the food industry (Denys, Van Loey, & Hendrickx, 2000). Moreover, POD is the most thermally resistant enzyme in vegetables and, for this reason it is usually used as an indicator of heat treatments in food processing. Consequently, POD has been widely used as a biological indicator of the effectiveness of blanching (Bahçeci, Serpen, Gökmen, & Acar, 2005).

The objective of our research was to partially purify and characterise POD from red alga *Mastocarpus stellatus* and to determine its kinetic parameters and thermal stability, in order to maximise the quality and minimize the economic and nutritional loss induced by this enzyme during the storage or processing of the seaweed.

2. Materials and methods

2.1. Plant material and growth conditions

Fresh, organically grown *M. stellatus* fresh seaweed (Rhodophyta), was kindly supplied at commercial maturity by Porto-Muiños (Cambre, A Coruña, Spain). Samples were transported to the laboratory and stored at 4 °C until they were used as a POD source.

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 of sodium phosphate buffer (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. Protein determination

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

2.4. POD extraction

Seaweed POD was extracted using the method described by Núñez-Delicado, Sánchez-Ferrer, García-Carmona, and López-Nicolás (2005). All extractions were performed in triplicate as explained below.

Seaweed (25 g) was washed and homogenised for 3 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, 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 (Núñez-Delicado, Bru, Sánchez-Ferrer, & García-Carmona, 1996). 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 an enzyme source, was stored at −20 °C.

2.5. Enzymatic activity

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 was kept at 25 °C and contained 20 μg/ml of protein, 100 mM sodium acetate buffer (pH 5.0), 3 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.

2.6. Optimum pH

The optimum pH profile was performed in the standard reaction medium, which consisted of 100 mM sodium acetate buffer (pH 3.0–5.0), 100 mM sodium phosphate buffer (pH 6.0–7.5) and 100 mM sodium borate buffer (pH 8.0–9.0), in both the absence and in the presence of 1 mM sodium dodecyl sulphate (SDS).

In the SDS standard assay, samples contained the above mixture and increasing concentrations of SDS (0–1 mM) in the cuvette.

In the reducing agents standard assay, samples contained the above mixture and increasing concentrations (0–2 mM) of ascorbic acid, L-cysteine or metabisulphite in the cuvette.

To determine the kinetic parameters of POD, the effect of ABTS and H_2O_2 concentration on the enzymatic activity was studied. To study the effect of ABTS concentration, it was increased from 0 to

9 mM in the standard reaction medium. In the case of H_2O_2, its concentration was increased from 0 to 10 mM in the standard reaction medium, at a fixed ABTS concentration (3 mM).

2.7. pH stability

The enzyme was incubated in 100 mM sodium acetate buffers pH 4.5, 5.0 or 5.5 at 4 °C for 60 min. Residual activity was determined at pH 5.0.

2.8. Optimum temperature

The optimum temperature was determined by measuring the enzymatic activity between 20 and 60 °C with 5 °C increments, using the optimum pH. Temperature was controlled using a TCC-Controller, Shimadzu Corporation (Mod. TCC-240A).

2.9. Thermal stability

The enzyme solutions (in Eppendorf tubes) were incubated in a circulating water bath, Julabo Shake Temp SW 22, at different temperatures (30, 35, 40 and 50 °C) for different times (up to 40 min). After heating, samples were cooled in ice water and assayed immediately at 25 °C.

Each sample was assayed in triplicate and the mean was plotted.

3. Results and discussion

POD was extracted by a phase-partitioning method with the detergent Triton X-114 (Sánchez-Ferrer, Bru, & García-Carmona, 1989a). This detergent has a cloud point at 23 °C, which allows it to be used in a temperature range compatible with protein stability. During phase separation induced by incubation at 37 °C, most of the detergent, plastidic pigments, phospholipids, phenolics, and very hydrophobic membrane proteins migrate to the lower detergent-rich phase, whereas polar phenolics and many other membrane proteins migrate to the upper aqueous detergent-poor phase (Cabanes, Escribano, Gandía-Herrero, García-Carmona, & Jiménez-Atiénzar, 2007). 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. The same method has been used to separate hydrophobic proteins (Bordier, 1981) and remove phenolic compounds (Núñez-Delicado et al., 1996) in addition to chlorophylls (Sánchez-Ferrer, Villalba, & García-Carmona, 1989b).

The enzymatic activity of POD extracted from the red alga *M. stellatus* was studied using ABTS as the H-donor and H_2O_2 as the acceptor. The pH is a determining factor in the expression of enzymatic activity. Fig. 1 shows the pH profile for the oxidation of ABTS by POD (open circles) in a pH range from 3.0 to 9.0, with 5.0 being the optimum. This optimum pH was similar to that obtained for PODs from several sources (Rodríguez-López et al., 2000a; Serrano-Martínez et al., 2008). However, the optimum pH of POD depends on the H-donor used in the activity assay. In some cases, guaiacol has been used as substrate (Chisari, Barbagallo, & Spagna, 2007, 2008), while in others ABTS has been used (Duarte-Vázquez, García-Almendárez, Regalado, & Whitaker, 2000; Rodríguez-López et al., 2000a). Incubating the enzyme for 60 min at pH values 4.5, 5.0 and 5.5 did not modify its enzymatic activity, which was always measured at pH 5.0 (data not shown). The stability of POD was only studied at pH values 4.5, 5.0 and 5.5, because at other pH values the enzymatic activity was shown to be very low.

The activating or inhibiting effect of SDS on different enzymes, including PPO and POD, has been widely described. In the case of POD, SDS showed an inhibitory effect in many cases (Fortea, López-Miranda, Serrano-Martínez, Carreño, & Núñez-Delicado, 2009; Nazari, Mahmudi, Shahrooz, Khodafarin, & Moosavi-Movahedi, 2005).

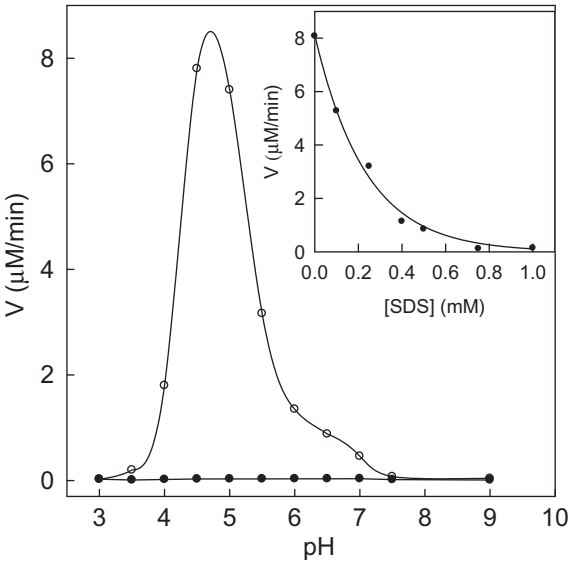

Fig. 1. Effect of pH on *Mastocarpus stellatus* seaweed POD activity in the absence (○) or in the presence of 1 mM SDS (●). (Inset) Effect of SDS concentration.

In order to study, the effect of SDS on the POD from *M. stellatus* seaweed, the pH profile was analysed in the presence of 1 mM SDS (Fig. 1, closed circles), the enzyme being found to be inactivated at all the studied pHs. A similar effect has previously been described for horseradish POD (Nazari et al., 2005) and *Crimson Seedless* table grape POD (Fortea et al., 2009).

Moreover, the effect of SDS depended on surfactant concentration, as shown in Fig. 1, inset. The enzymatic activity decreased as SDS concentration increased, with total inactivation being reached at 1 mM SDS. This observed inactivation was due to the conformational changes produced in the protein by the interaction with the detergent molecules. These results contrast with those described for PPO obtained from a variety of sources, where the changes produced by the SDS interaction had an activating effect (Cabanes et al., 2007; Fortea et al., 2009; Núñez-Delicado et al., 2005).

Kinetic parameters, maximum rate (V_m) and Michaelis constant (K_M), of *M. stellatus* seaweed POD were determined at pH 5.0. The apparent kinetic parameters (V_m and K_M) were determined by fitting the experimental points to the Michaelis–Menten equation using the data obtained at pH 5.0. Fig. 2 shows variations in initial velocity *versus* substrate concentration. The steady state rate increased with ABTS concentration (Fig. 2). The K_M value for ABTS was found to be 13 mM and the V_m value was 40 μM/min. This K_M value obtained for ABTS (13 mM) was much higher than that described for turnip POD (0.4 mM; Duarte-Vázquez et al., 2000), pepper POD (0.5 mM; Serrano-Martínez et al., 2008), Crimsom Seedles grape (0.8 mM; Fortea et al., 2009), and acidic horseradish POD (4 mM; Hiner, Hernández-Ruiz, Arnao, García-Cánovas, & Acosta, 1996).

In addition, when the H_2O_2 concentration was increased over a range of 0–8 mM (Fig. 2, inset) at a fixed concentration of ABTS (3 mM), the activity increased at first before falling gradually at higher concentrations. The ABTS concentration should be below K_M because the high ε of $ABTS^{+\cdot}$ hindered measurement at higher concentrations. Fig. 2 inset, shows a typical kinetic profile of substrate inhibition, which can be kinetically analysed by non-linear

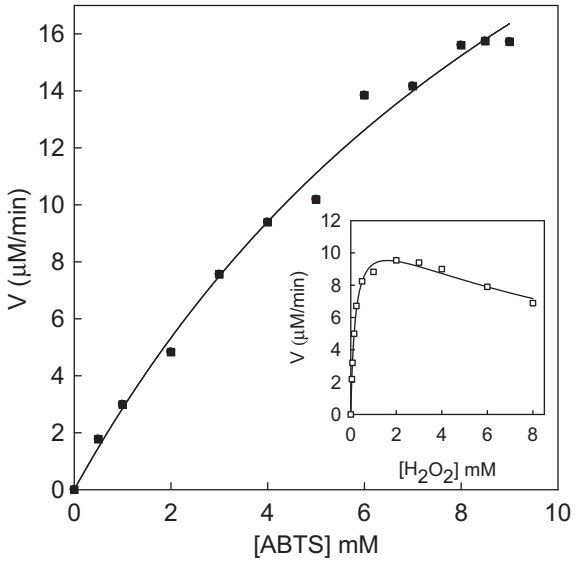

Fig. 2. Effect of ABTS concentration on *Mastocarpus stellatus* seaweed POD activity. (Inline) Effect of H_2O_2 concentration.

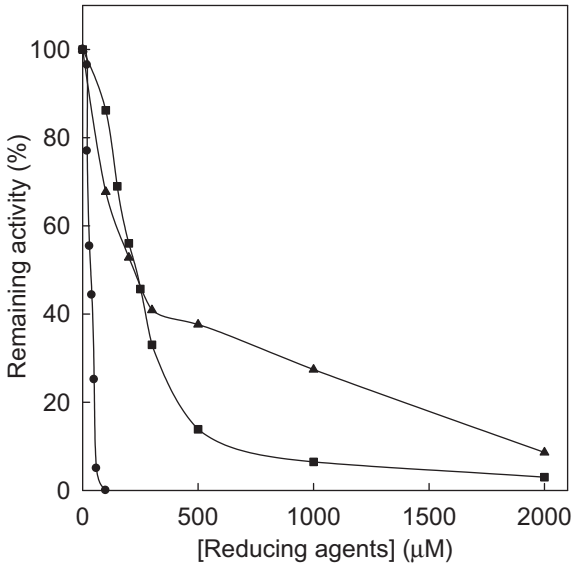

Fig. 3. Inhibitory effect of reducing agents. The reaction medium at 25 °C contained. 100 mM sodium acetate buffer (pH 5.0), 2 mM H_2O_2, 3 mM ABTS, 0.2 mM tropolone and 20 μg/ml enzyme and reducing agents concentrations ranging 0–2 mM (L-cysteine (●), metabisulphite (▲) or ascorbic acid (■)).

regression fitting of the experimental points to the following equation (Marquard, 1963).

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

where k_{si} is the substrate inhibition constant. The kinetic parameters, V_m, K_M and k_{si}, obtained were 12 μM/min, 0.2 mM and 12.3 mM, respectively. This kinetic profile agrees with those obtained for horseradish, asparagus and turnip peroxidases (Duarte-Vázquez et al., 2000; Hiner et al., 1996), in which an inhibition by substrate concentration was described for H_2O_2, but contrasts with those described for pepper and crimsom seedless grape POD (Fortea et al., 2009; Serrano-Martínez et al., 2008) in which the activity increased with H_2O_2 concentration to reach saturation.

The K_M value obtained for H_2O_2 (0.2 mM) was lower than those obtained for POD from pepper (1.3 mM, Serrano-Martínez et al., 2008), turnip (0.8 mM, Duarte-Vázquez et al., 2000) or grape (0.4 mM; Fortea et al., 2009), but was similar to that described for melon POD (0.2 mM, Rodríguez-López et al., 2000a).

To further characterise the red alga POD, its inhibition by reducing agents was studied. Fig. 3 shows the effect of various reducing agents (ascorbic acid, L-cysteine and metabisulphite) on red alga POD activity, using ABTS as substrate. L-Cysteine appeared to be the most effective inhibitor (Fig. 3; closed circles), an ability that has been extensively described for both POD (Serrano-Martínez et al., 2008) and PPO (Liu et al., 2007). Ascorbic acid, another reducing agent, acts as antioxidant rather than as an enzyme inhibitor because it reduces the ABTS radical to its original form (Fig. 3; closed squares), an effect that has also been previously described in the literature (Serrano-Martínez et al., 2008). The inhibition produced by metabisulphite (thiol compound) was lower (Fig. 3; closed triangles), perhaps as a result of an additional reaction taking place with the ABTS radical to form stable products and/or binding to the active centre of the enzyme as in the case of metabisulphite with red pepper POD (Serrano-Martínez et al., 2008) and polyphenol oxidase (Valero, Varón, & García-Carmona, 1992).

The optimum temperature of activity for *M. stellatus* POD was found at 25 °C. Temperatures higher than 30 °C in the assay medium caused a progressive decrease of activity until 95% inactivation was observed at 55 °C (data not shown).

To complete the study of *M. stellatus* POD, its thermal stability was studied. The semi-log plots of the residual activity of POD *versus* heating time were linear at all the temperatures studied (Fig. 4), which is consistent with inactivation by means of 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 (Fig. 4). This result is in accordance with those obtained for other PODs from pepper (Serrano-Martínez et al., 2008), potato or carrot (Anthon & Barrett, 2002) and grape (Fortea et al., 2009). From the slopes of these lines, the inactivation rate constants (k) were calculated by linear regression according to the equation (Anthon & Barrett, 2002):

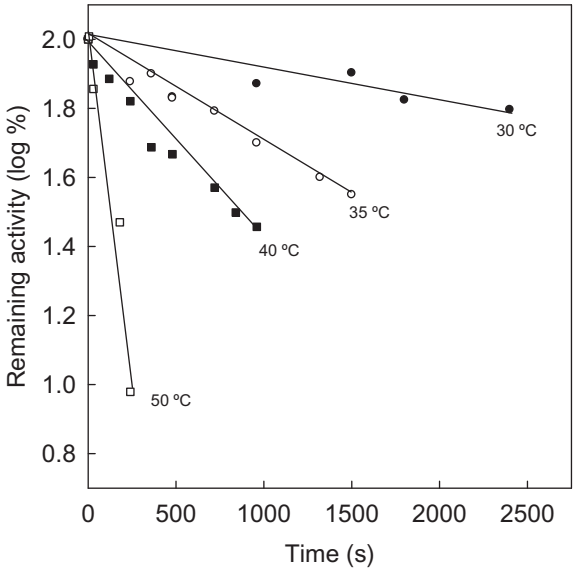

Fig. 4. Heat inactivation of *Mastocarpus stellatus* seaweed POD. Remaining POD activity versus heating time at 30 °C (●), 35 °C (○), 40 °C (■), 50 °C (□). The reaction medium contained 100 mM sodium acetate buffer (pH 5.0), 2 mM H_2O_2, 3 mM ABTS, 0.2 mM tropolone and 20 μg/ml enzyme.

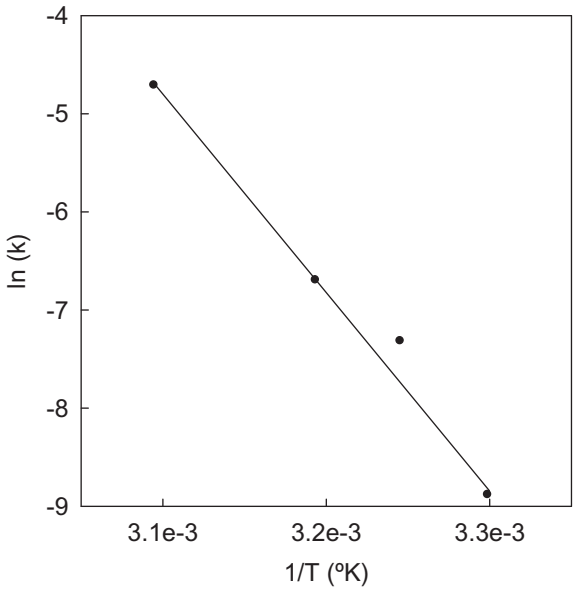

Fig. 5. Arrhenius plot of inactivation rates from POD.

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

where A_0 is the initial enzyme activity and A is the activity after heating for time t. When the k values obtained were plotted in an Arrhenius plot (Fig. 5), a simple linear fit was obtained. The activation energy (E_a) was calculated from the slope of the Arrhenius plot by the equation (Anthon & Barrett, 2002)

$$\ln(k) = -E_a/RT + c$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is temperature in K. This linear fit contrasts with the curvature obtained in other vegetables, including potato and carrot PODs (Anthon & Barrett, 2002), green bean, pea seed lipoxygenases, and grape (Fortea et al., 2009) but agrees with that obtained for pepper POD (Serrano-Martínez et al., 2008). The explanation for this linearity in the Arrhenius plot is that the inactivation in *M. stellatus* seaweed POD occurs through a unique mechanism dependent on temperature, such as protein unfolding. In contrast, in the cases in which a curvature in the Arrhenius plot is observed, the explanation is that the inactivation occurs through more than one mechanism, each with its own temperature dependence and the overall temperature dependence is simply the sum of the individual processes.

Table 1 reviews recent kinetic data on POD thermal inactivation in some vegetables. In the case of *M. stellatus* seaweed POD, the value obtained for E_a (121.6 kJ/mol) was about half that obtained for grape POD (271.9 kJ/mol; Fortea et al., 2009), four times lower than those obtained for potato (478 kJ/mol) or carrot (480 kJ/mol) PODs (Anthon & Barrett, 2002), but was similar to that obtained for "Amarillo" melon (160 kJ/mol; Chisari et al., 2008), "Charantais" melon (86 kJ/mol; Chisari et al., 2008), pepper PODs (151 kJ/mol; Serrano-Martínez et al., 2008), "Elsanta" and "Madame Moutot" strawberry PODs (96 and 74 kJ/mol, respectively; Chisari et al., 2007). The range of temperatures required for the inactivation of *M. stellatus* seaweed POD was 30–50 °C, similar to that required for pepper POD (30–60 °C; Serrano-Martínez et al., 2008), but lower than that required for *Crimson Seedless* grape (60–80 °C; Fortea et al., 2009), potato (67–85 °C) and carrot (70–84 °C) (Anthon & Barrett, 2002), strawberry (50–80 °C; Chisari et al., 2007), and melon PODs (40–70 °C; Chisari et al., 2008). These results indicated that *M. stellatus* seaweed POD is less thermostable than *crimson seedless* grape, strawberry, melon, potato and carrot PODs, but similar to pepper POD.

In some cases, inactivation is expressed 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 for *M. stellatus* seaweed POD was 48.4 °C, which is similar to that obtained for pepper POD (44.5 °C, Serrano-Martínez et al., 2008), but half that obtained for *Crimson Seedless* grape (75 °C; Fortea et al., 2009), potato and carrot (80 °C; Anthon & Barrett, 2002), melon (70 °C; Chisari et al., 2008), and strawberry PODs (80 °C; Chisari et al., 2007).

In conclusion, this paper presents a detailed kinetic study of *M. stellatus* seaweed POD isolated using TX-114. The enzyme POD thus isolated was inactivated by the anionic detergent SDS. The reducing agents L-cysteine, ascorbic acid and metabisulphite also inhibited this enzyme. A thermal stability study showed that this POD is a very thermolabile enzyme compared with PODs extracted from other plant sources, showing a D 5 min of 48.4 °C. The thermostability of POD is very important, because this enzyme may have very negative effect on the colour and flavour of vegetables during storage.

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Table 1
Published kinetic parameters for POD thermal inactivation.

References	Product	Temperature range (°C)	E_a (kJ/mol)	D value (°C)
Serrano-Martínez et al. (2008)	Pepper	30–60	151	44.5
Chisari et al. (2008)	Melon	40–70	"Charantais": 86 "Amarillo" 160	70
Chisari et al. (2007)	Strawberry	50–80	"Elsanta" 96 "Madame Moutot" 74	80
Fortea et al. (2009)	Grape	60–80	271.9	75
Anthon and Barrett (2002)	Potato	67–85	478	80
Anthon and Barrett (2002)	Carrot	70–84	480	80

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