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Characterization of Polyphenol Oxidase and Peroxidase and Influence on Browning of Cold Stored Strawberry Fruit

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Polyphenol oxidase and peroxidase were extracted from two different varieties of strawberry fruit (*Fragaria x ananassa* D, cv. 'Elsanta' and *Fragaria vesca* L, cv. 'Madame Moutot') and characterized using reliable spectrophotometric methods. In all cases, the enzymes followed Michaelis−Menten kinetics, showing different values of peroxidase kinetics parameters between the two cultivars: K_m = 50.68 ± 2.42 mM ('Elsanta') and 18.18 ± 8.79 mM ('Madame Moutot') mM and V_{max} = 0.14 ± 0.03 U/g ('Elsanta') and 0.05 ± 0.01 U/g ('Madame Moutot'). The physiological pH of fruit at the red ripe stage negatively affected the expression of both oxidases, except polyphenol oxidase from 'Madame Moutot' that showed the highest residual activity (68% of the maximum). Peroxidase from both cultivars was much more thermolabile as compared with PPO, losing over 60% of relative activity already after 60 min of incubation at 40 °C. The POD activation energy was much lower than the PPO activation energy ($\Delta E^{\ddagger}$ = 97.5 and 57.8 kJ mol^{-1} for 'Elsanta' and 'Madame Moutot', respectively). Results obtained from D-glucose and D-fructose inhibition tests evidenced a decreasing course of PPO and POD activities from both cultivars as the sugar concentration in the assay medium increased. Changes in CIE L^*, a^*, b^*, chroma, and hue angle values were taken as a browning index of the samples during storage at 4 °C. A decrease in L^* was evident in both cultivars but more marked in 'Elsanta'. PPO and POD activities from cv. 'Elsanta' were very well-correlated with the parameter L^* (r^2 = 0.86 and 0.89, respectively) and hue angle (r^2 = 0.85 and 0.93, respectively). According to these results, the browning of the fruit seemed to be in relation to both oxidase activities.

KEYWORDS: Strawberry; polyphenol oxidase; peroxidase; browning; anthocyanins

INTRODUCTION

The strawberry, among the berry species, has obtained the best commercial development in recent years (*1*). Unfortunately, this fruit has a very short post-harvest life, due to its relative high metabolic activity and high sensitivity to fungal attack. Furthermore, during handling, storage, and marketing, it is highly susceptible to physical damage leading to disruption of its cellular structure and consequently a speed up of softening and browning phenomena.

Browning of damaged tissues of fresh fruits and vegetables mainly occurs from the oxidation of phenolic compounds and contributes significantly to quality loss (*2*). In particular, the primary enzyme responsible for the browning reaction is polyphenol oxidase (PPO; EC 1.14.18.1) (*3*). In the presence of oxygen, this copper enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity) (*4*). These, in turn, are polymerized to undesirable brown, red, or black pigments (*5*). In plants, PPO is predominantly located in the chloroplast thylakoid membranes, and its phenolic substrates are mainly located in the vacuoles, but upon any cell-damaging treatment, the enzyme and substrates may come into contact, leading to rapid oxidation of phenols (*6*).

Peroxidase (POD; EC 1.11.1.7) is another oxidoreductase enzyme involved in enzymatic browning since diphenols may function as reducing substrates in its reaction (*7*). The involvement of POD in browning is reported by many researchers (*8*−*10*), 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 catalyzes the cross-linking between ferulic acid substituents of pectins (*11*), and a clear correlation has been found between its activity and the synthesis of lignin and suberin polymers (*12*).

Much of the literature reports the characterization of oxidative enzymes from various fruits and vegetables, such as apples (*13*), grapes (*14*), pears (*15*), eggplants (*16*), and strawberries (*17*), but no clear correlations were found between degradative activities and browning of samples. The aim of this work was to extract and characterize polyphenol oxidase and peroxidase from strawberry to determine kinetic parameters, optimum conditions of pH and temperature, thermal stability, and inhibitor

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effects by D-glucose and D-fructose. Furthermore, storage tests at 4 °C were performed with whole fruit to evaluate the variations of physicochemical properties and to establish possible relationships between oxidase activities and browning of fruit.

MATERIALS AND METHODS

Plant Materials. Strawberries belonging to groups *Fragaria x ananassa* D, cv. 'Elsanta' and *Fragaria vesca* L, cv. 'Madame Moutot' were all obtained on the same day, on the day of harvest from local producers in Catania and Maletto (Sicily, Italy). The cultivar 'Elsanta' is widely present in Italian national markets because of its form, high productivity, sturdiness, and long shelf life. The cultivar 'Madame Moutot', originally imported from France, represents instead a typical product of Sicilian strawberry cultivation, restricted to the slopes of Mount Etna. Fruits were harvested at commercial maturity (25−30 days after anthesis), when they were at full size and at about 90% full red color. They were sorted on the basis of size and absence of physical damage and divided randomly into lots of 10 (replicates) ca. 200 g of fruit (average weight of single fruit: 19.4 ± 0.8 and 18.5 ± 1.1 g for 'Elsanta' and 'Madame Moutot', respectively). Fruits were placed in cold storage (4 °C) immediately after harvest and kept for 18 h before beginning enzyme extraction and analyses. The storage kept the freshly harvested fruit at 4 °C and 95% RH, in transparent perforated polystyrene baskets with a capacity of 500 g of strawberries, wrapped with perforated PVC plastic film (25 holes of 5 mm diameter per package, 0.02 mm film thickness), in the same way that they are usually exposed in supermarket displays. Ten fruits of each species were sampled for physicochemical analyses and enzymatic assays at days 0, 3, 7, and 10. Five hundred grams of freshly harvested fruits from each species was utilized for enzyme characterization.

Sample Preparation. A 200 g strawberry sample was passed through a juice centrifuge, sieved (0.5 mm diameter) to remove seeds, and homogenized using a Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) homogenizer set for 60 s.

PPO and POD Extraction and Assay. Twenty grams of homogenate was added to 40 mL of cold acetone (−20 °C) and continuously stirred for 10 min. The homogenate was filtered through Whatman No. 42 paper under vacuum on a Buchner funnel; the acetone powder, after elimination of the acetone under vacuum, was collected and suspended in 30 mL of 0.1 M citrate phosphate buffer pH 7.5 and kept overnight at 4 °C, before again being filtered through Whatman No. 42 paper under vacuum on a Buchner funnel. The clear solution was ultrafiltered in a Millipore stirred cell with a 10 kDa membrane (Millipore 8050, Milan, Italy) and utilized as the crude enzymatic extract.

The enzymatic assay was performed according to a reliable spectrophotometric method, using MBTH to trap the enzyme generated ortoquinone (*18*, *19*). PPO activity was assayed spectrophotometrically at 505 nm using 3,4-dihydroxyphenyl acetic acid as a phenolic substrate with MBTH. The standard reaction mixture contained 0.9 mL of 40 mM phenolic substrate, 0.1 mL of 2% (w/v) MBTH in methanol, 0.05 mL of DMF, 1.5 mL of 50 mM sodium acetate buffer pH 7.0, and 0.5 mL of enzymatic extract. The reaction was stopped at different times with 0.5 mL of 5% H_2SO_4. The blank was prepared by inverting the order between enzymatic extract and H_2SO_4. One unit of PPO activity was defined as the amount of enzyme that produces 1 μmol of adduct per min at 25 °C under the conditions previously described.

POD activity was determined spectrophotometrically as the change in absorbance at 470 nm. The reaction mixture contained 2 mL of 0.01 M citrate phosphate buffer (pH 7.0) containing 1.0% (v/v) guaiacol, 0.25 mL of 32 mM H_2O_2, and 0.1 mL of enzyme extract (*20*). One guaiacol unit (U) is defined as the amount of enzyme that oxidizes 1 μmol of guaiacol per minute at 25 °C and pH 7.0 under the conditions described previously.

The protein content was determined according to the Bradford Bio-Rad protein assay using bovine serum albumin as a standard (*21*).

Kinetic Properties. PPO and POD activities were measured with their specific substrates (DOPAC and hydrogen peroxide, respectively) in order of increasing molarity (up to 80 mM for DOPAC and 512 mM for hydrogen peroxide). Enzyme behavior (at pH 7.0 and 25 °C) was explained by the Michaelis−Menten equation, while kinetic parameters (K_m and V_{max}) were calculated by hyperbolic regression analysis (*22*).

Optimum pH and Temperature. PPO and POD activities were determined in a pH range of 3.0−8.0 in 50 mM citrate phospate buffer, using DOPAC (40 mM) and hydrogen peroxide (32 mM) as substrates. Then, tests were carried out at the pH producing maximum activity to find the optimal temperature; PPO and POD activities were assayed at various reaction temperatures as controlled by a circulation water bath. The temperature was varied over the range of 4−70 ± 0.1 °C. DOPAC (40 mM) and hydrogen peroxide (32 mM) were used as substrates.

Thermal Stability. The enzyme solutions in Eppendorf tubes were incubated in a water bath at four different temperatures (50, 60, 70, and 80 °C) for different times, up to 120 min. PPO activity was determined at 25 °C and pH 7.0, using DOPAC (40 mM) as a phenolic substrate. POD activity was determined at 25 °C and pH 7.0, using hydrogen peroxide (32 mM) as a substrate. The percentage of residual enzymatic activity was calculated by comparison with unheated enzyme (*23*).

Inhibition Tests. D-Glucose and D-fructose at different concentrations (0.02, 0.06, 0.1, 0.5, 1, 2, 3, 4, and 5 M) were dissolved in the assay medium, and PPO and POD activities were measured at 25 °C and pH 7.0, to determine inhibitor effects of sugars on enzymatic activities.

Physicochemical Properties of Strawberry Samples. Each pulp sample was blended with a standard blender (Solac 850, Vitoria, Spain), and the obtained juice was used for chemical analysis. Titratable acidity (TA) was quantified by titrating 10 mL of strawberry juice with 0.1 N NaOH to an endpoint of pH 8.1 (Metrohm 716 DMS titrator, Herisau, Switzerland) and expressed as g of citric acid 100 mL^{-1} (*24*). The total soluble solid (TSS) content was measured with a digital refractometer (Abbe 1S, Milan, Italy) and expressed as °Bx at 20 °C. The pH was measured with the same equipment used for measuring TA. Anthocyanins were estimated by a pH differential method (*25*). Absorbance was measured in a Varian Cary 1E (Milan, Italy) spectrophotometer at 510 and at 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyanidin-3-glucoside of 29 600. Results were expressed as micrograms of cyanidin-3-glucoside equiv per gram of fresh weight. The juice color was determined with a compact tristimulus chromameter (Minolta CR-300, Ramsey, NJ) with an 8 mm Ø viewing aperture, white plate reference ($Y = 94.3$; $x = 0.3142$; $y = 0.3211$), and C illuminant (CIE, 2° observer). Readings were expressed as L^*, a^*, and b^* parameters. Chroma $[(a^{*2} + b^{*2})^{1/2}]$ and hue angle $[\tan^{-1}(b^*/a^*)]$ were calculated.

Statistical Analysis. All determinations were conducted 3 times at least. Analysis of variance (ANOVA) of the data was evaluated by the Statistical Analysis System (SAS version 9.0). Duncan's multiple range test was employed to determine the statistical significance of the differences between the means ($p \leq 0.05$).

RESULTS AND DISCUSSION

Kinetics Properties. PPO and POD kinetics parameter determination was carried out on extracts from 'Elsanta' and 'Madame Moutot' cultivars (**Figure 1a**,**b**). Relative to polyphenol oxidase, in both cultivars, the enzyme followed Michaelis−Menten kinetics, showing very similar K_m and V_{max} values for the DOPAC substrate: $K_m = 4.78 \pm 1.87$ mM ('Elsanta') and 5.67 ± 2.20 mM ('Madame Moutot') mM and $V_{max} = 0.02 \pm 0.005$ U/g ('Elsanta') and 0.03 ± 0.007 U/g ('Madame Moutot'). Also, peroxidase extracted from strawberry samples showed Michaelis−Menten kinetics but with different values of kinetics parameters between the two cultivars: $K_m = 50.68 \pm 2.42$ mM ('Elsanta') and 18.18 ± 8.79 mM ('Madame Moutot') mM and $V_{max} = 0.14 \pm 0.03$ U/g ('Elsanta') and 0.05 ± 0.01 U/g ('Madame Moutot').

pH Optimum. The strawberry PPO had a maximum activity at pH 4.0 in cultivar 'Elsanta' and at pH 4.5 in cultivar 'Madame Moutot' (**Figure 2**) but retained its residual activity over 60%

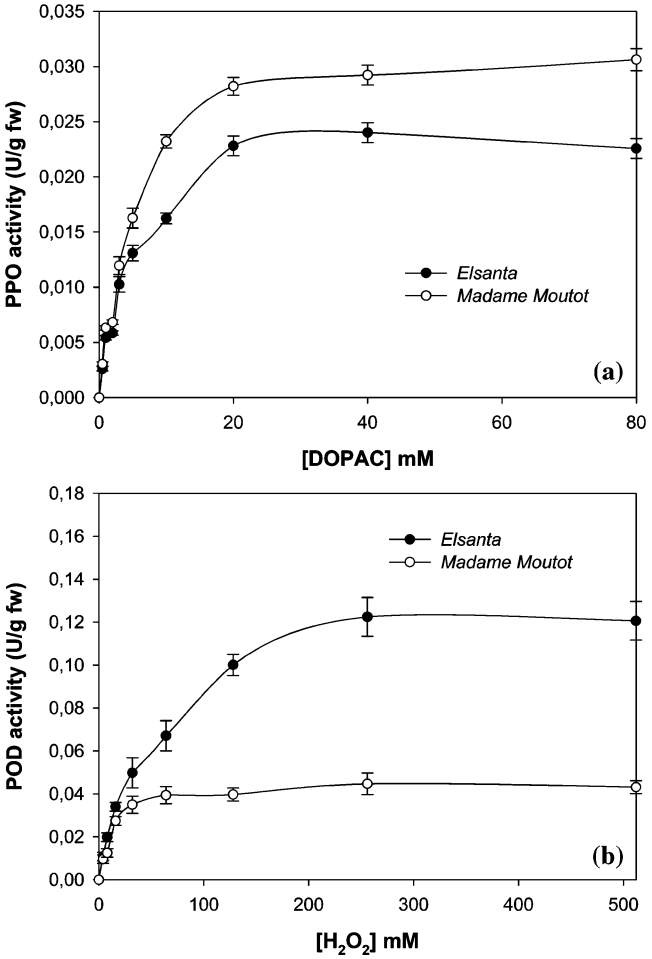

Figure 1. (**a**) Hyperbolic regression results for strawberry PPO (±95% confidence intervals). (●) 'Elsanta' (V_{max} = 0.02 ± 0.005 U/g; K_m = 4.78 ± 1.87 mM) and (○) 'Madame Moutot' (V_{max} = 0.03 ± 0.007 U/g; K_m = 5.67 ± 2.20 mM). (**b**) Hyperbolic regression results for strawberry POD (±95% confidence intervals). (●) 'Elsanta' (V_{max} = 0.14 ± 0.03 U/g; K_m = 50.68 ± 2.42 mM) and (○) 'Madame Moutot' (V_{max} = 0.05 ± 0.01 U/g; K_m = 18.18 ± 8.79 mM).

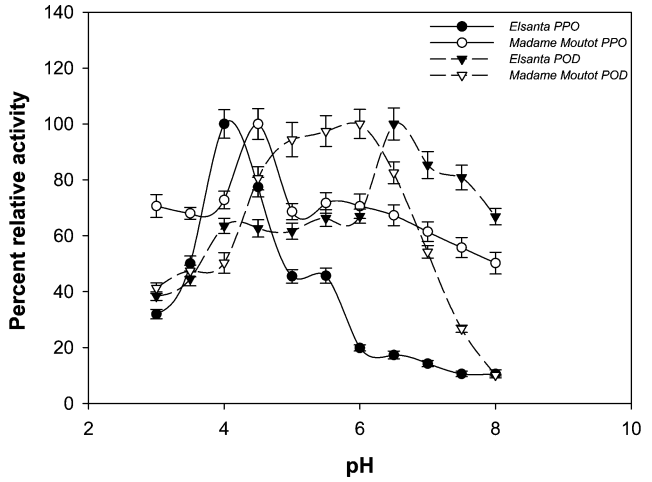

Figure 2. Effect of pH on strawberry PPO and POD activity.

in the pH range of 3.0−6.0 in 'Madame Moutot' while rapidly decreasing in 'Elsanta' until there was a 20% residual activity at pH 6.0. The physiological pH in red ripe fruits was 3.5 in both cultivars, in which condition the PPO relative activity was 50% in 'Elsanta' and 68% in 'Madame Moutot'. With regard to peroxidase, the maximum activity was found at pH 6.5 in

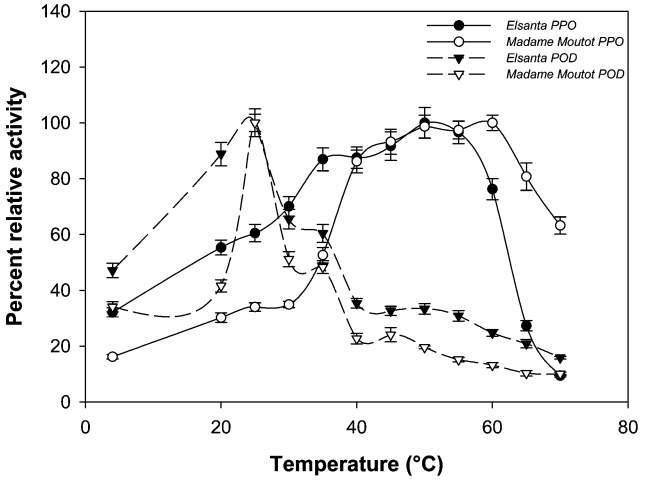

Figure 3. Effect of temperature on strawberry PPO and POD activity.

'Elsanta' and 6.0 in 'Madame Moutot' (**Figure 2**). More acidic pH values caused a decrease of activity, probably due to enzyme instability at these pH values. At a strawberry physiological pH value of 3.5, the residual activity was similar between the cultivars (44% in 'Elsanta' and 47% in 'Madame Moutot'). Results indicated that the red ripe fruit condition negatively affected the expression of both oxidases, except polyphenol oxidase from 'Madame Moutot' that showed the highest residual activity (68% of the maximum).

Optimum Temperature. The optimum temperature of activity for strawberry PPO was 50 °C in 'Elsanta' and 65 °C in 'Madame Moutot' (**Figure 3**). Results agree with those reported in the literature relative to PPO extracted from different sources (*26*−*28*). PPO retained most of its activity (more than 80% of the maximum) over the temperature range of 40−65 °C. On the other hand, at the cold storage temperature (4 °C), PPO resulted in a very low residual activity (32 and 16% of the maximum in 'Elsanta' and 'Madame Moutot', respectively). Relative to POD, the maximum activity was found at 25 °C in both cultivars (**Figure 3**). Temperatures higher than 30 °C in the assay medium caused a progressive decrease of activity until 90% inactivation at 70 °C. The cold storage temperature (4 °C) produced a slighter inactivation, leading to 47 and 34% POD residual activity in 'Elsanta' and 'Madame Moutot', respectively.

Thermal Stability. The thermostability profiles of PPO extracts from 'Elsanta' and 'Madame Moutot' are shown in **Figure 4a**,**b**. PPO from 'Elsanta' showed a good stability during 60 min of incubation at 50 °C, while at 60 °C, almost a complete inactivation (95% loss of activity) after 20 min of incubation was noticed. 'Madame Moutot' PPO resulted in being much more thermostable than 'Elsanta' above all at high temperatures, showing over 40% of residual activity after 2 h of incubation at 60 °C. The time required to halve the activity was 77 min at 50 °C, 4.5 min at 60 °C, 2.6 min at 70 °C, and 1.7 min at 80 °C for 'Elsanta' and 153 min at 50 °C, 93 min at 60 °C, 38 min at 70 °C, and 9.8 min at 80 °C for 'Madame Moutot'. PPO is not a very heat-stable enzyme as compared to other enzymes responsible for quality degradation of fruit and vegetables (*29*); however, strawberry PPO showed good resistance at temperatures (50 °C) much higher than those utilized for the storage of fresh produce (from 4 to 25 °C).

Thermostability profiles of POD from 'Elsanta' and 'Madame Moutot' are shown in **Figure 5a**,**b**. Peroxidase from both cultivars resulted in being much more thermolable as compared with PPO, losing 60−80% of relative activity after 60 min of incubation at 50 °C. However, the commercial cultivar 'Elsanta'

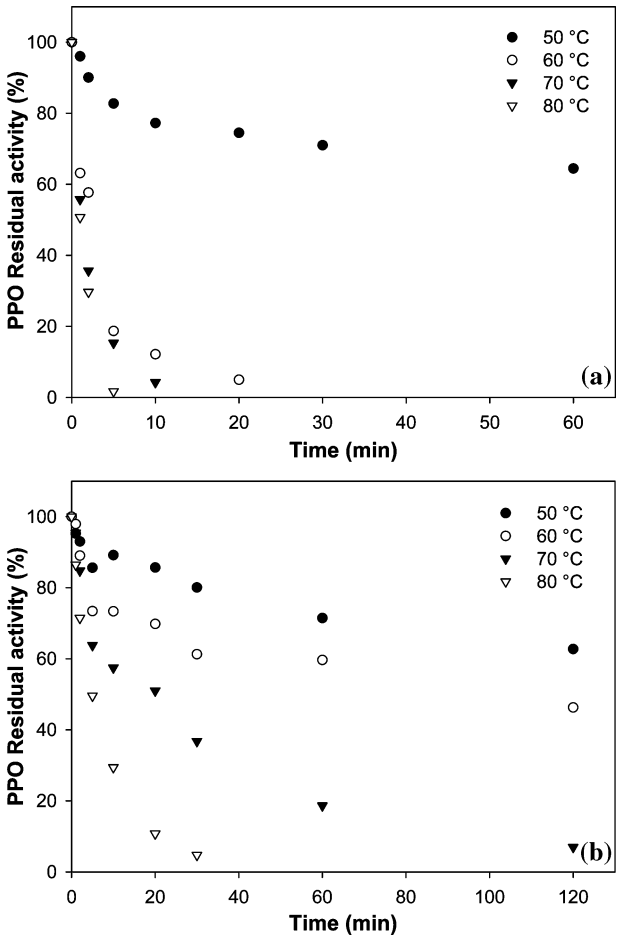

Figure 4. (**a**) Thermal stability of 'Elsanta' PPO and (**b**) thermal stability of 'Madame Moutot' PPO.

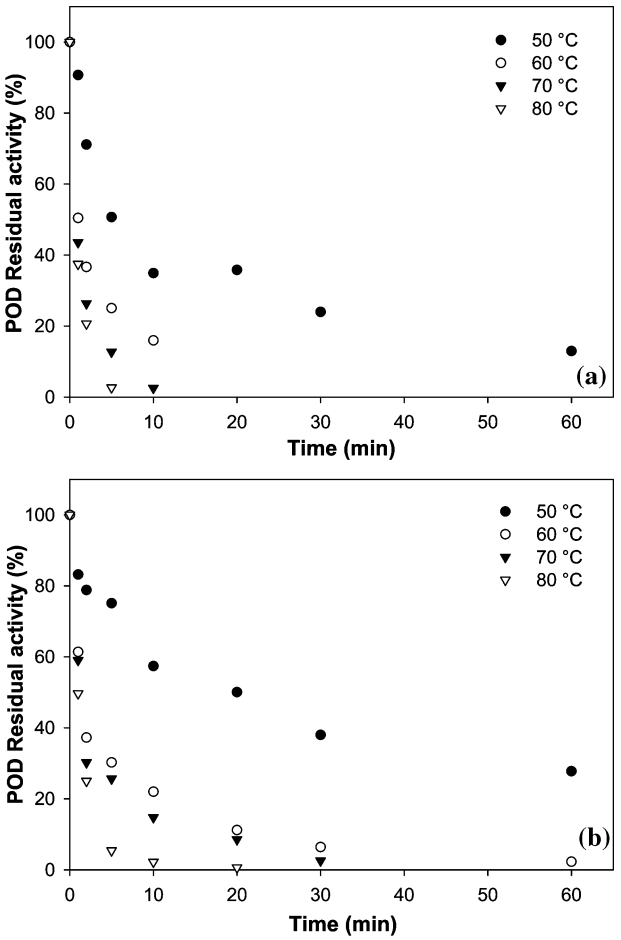

Figure 5. (**a**) Thermal stability of 'Elsanta' POD and (**b**) thermal stability of 'Madame Moutot' POD.

resulted in being more stable than the local cultivar 'Madame Moutot' in the temperature range of 50−80 °C. The time required to halve the activity was 18 min at 50 °C, 2.9 min at 60 °C, 1.8 min at 70 °C, and 1.4 min at 80 °C for 'Elsanta' and 29 min at 50 °C, 4 min at 60 °C, 1.3 min at 70 °C, and 0.7 min at 80 °C for 'Madame Moutot'.

The temperature dependence of the kinetic inactivation constant (k) was evaluated using the Arrhenius equation

$$\ln k = \ln k_0 - \Delta E^{\ddagger}/RT \quad (1)$$

as shown in **Figure 6**. The activation energy ($\Delta E^{\ddagger}$) for crude 'Elsanta' and 'Madame Moutot' PPO and POD heat inactivation was determined from results in **Figure 6**. Other activation parameters, namely, $\Delta G^{\ddagger}$ (Gibbs free energy for enzyme inactivation), $\Delta H^{\ddagger}$ (enthalpy change, a measure of the number of noncovalent bonds broken), and $\Delta S^{\ddagger}$ (entropy change, a measure of net enzyme and solvent disorder), were determined from the following equations as described previously (*30*):

$$\Delta G^{\ddagger} = RT \ln(kT/K_B h) \quad (1a)$$

$$\Delta H^{\ddagger} = \Delta E^{\ddagger} - RT \quad (1b)$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T \quad (1c)$$

where R (8.3145 J mol^{-1} K^{-1}) is the universal gas constant, K_B (1.3806 × 10^{-23} J K^{-1}) is the Boltzman constant, h (6.6261 × 10^{-34} J s) is Planck's constant, and T is the absolute temperature. Results for these analyses are reported in **Table 1**.

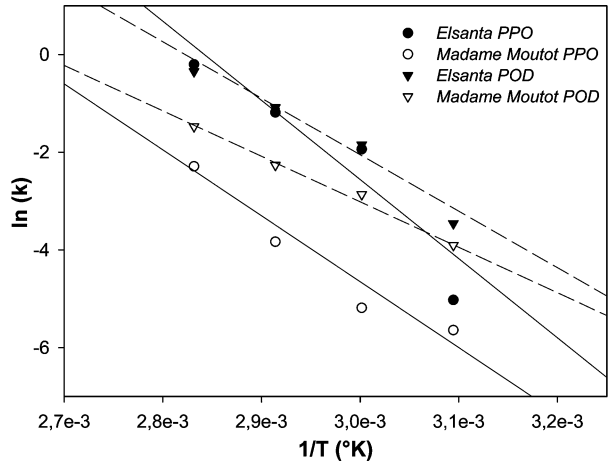

Figure 6. Arrhenius plot for heat inactivation of strawberry PPO and POD.

To summarize, $\Delta E^{\ddagger}$ was 145.6 kJ mol^{-1} for 'Elsanta' PPO heat inactivation as compared to 107.5 kJ mol^{-1} for 'Madame Moutot' PPO heat inactivation. POD activation energy resulted in being much lower than PPO (96.2 and 74.6 kJ mol^{-1} for 'Elsanta' and 'Madame Moutot', respectively). At temperatures of 50−80 °C, the average values of $\Delta H^{\ddagger}$ were 142.7 and 104.7 kJ mol^{-1} for 'Elsanta' and 'Madame Moutot' PPO, respectively, and 93.4 and 71.8 kJ mol^{-1} for 'Elsanta' and 'Madame Moutot' POD, respectively. Considering the higher values of $\Delta E^{\ddagger}$ and $\Delta H^{\ddagger}$ at all temperatures and the greater half-life values for polyphenol oxidase than those for peroxidase, it is possible to conclude that strawberry PPO is more thermostable than POD

Table 1. Transition State Parameters for Heat Inactivation of Crude 'Elsanta' and 'Madame Moutot' PPO and POD[a]

enzymatic extract	$\Delta E^{\ddagger}$[b] (kJ mol^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta G^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})
		'Elsanta'		
PPO	145.6	142.7 ± 0.9	373.4 ± 11.8	−681.2 ± 29.1
POD	96.2	93.4 ± 0.9	374.5 ± 10.4	−830.7 ± 19.2
		'Madame Moutot'		
PPO	107.5	104.7 ± 0.9	367.4 ± 10.4	−766.0 ± 21.5
POD	74.6	71.8 ± 0.9	371.8 ± 9.4	−886.9 ± 14.9

[a] Means ±SD for triplicate experiments. [b] $\Delta E^{\ddagger}$: Activation energy for crude PPO and POD heat inactivation; $\Delta G^{\ddagger}$: Gibbs free energy for enzyme inactivation; $\Delta H^{\ddagger}$: enthalpy change; and $\Delta S^{\ddagger}$: entropy change.

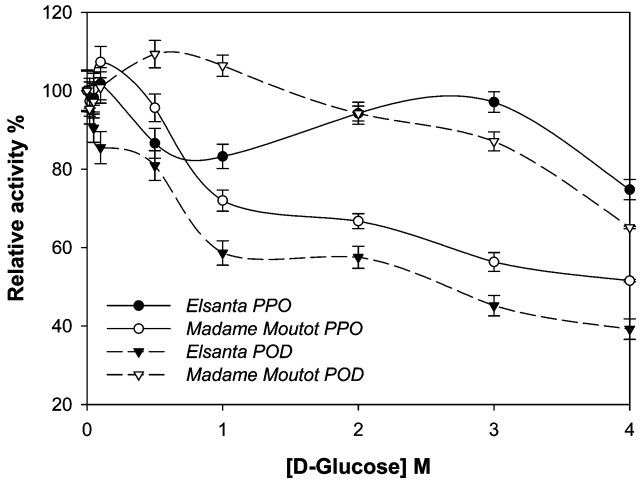

Figure 7. D-Glucose inhibition of crude strawberry PPO and POD.

over the range of temperatures studied. Furthermore, some differences between the cultivars were noticed. In particular, PPO and POD extracts from 'Elsanta' showed a much higher thermostability than those from 'Madame Moutot', in terms of thermal inactivation parameters and time required to halve the activity.

Sugar Inhibition. The effects of D-glucose as an enzymatic inhibitor are shown in **Figure 7**. The percentage of inhibition was compared with that of the control (100% activity). PPO and POD activities from both cultivars showed a decreasing course as the sugar concentration in the assay medium increased. The effect of D-glucose on crude 'Elsanta' PPO activity was similar in pattern to that of crude 'Madame Moutot' PPO activity. The concentration of D-glucose required in the assay medium to halve the activity was 3.05 and 1.84 M for 'Elsanta' and 'Madame Moutot' PPO, respectively. Peroxidase seemed to be less affected by D-glucose presence in the assay medium, especially in extracts from cultivar 'Madame Moutot', in which a slight activation up to 1 M sugar concentration was noticed.

The inhibition effects of D-fructose on strawberry PPO and POD are shown in **Figure 8**. Also, in this case, the increasing concentrations of sugar caused a progressive inactivation of both enzymes, which was a much more evident inhibition in strawberry polyphenol oxidase than in peroxidase. In fact, the concentration of D-fructose required to halve the activity was 4.05 and 3.15 M for 'Elsanta' and 'Madame Moutot' PPO, respectively, while it was 10.49 and 3.61 M for 'Elsanta' and 'Madame Moutot' POD, respectively. By the results obtained, it is possible to notice the better resistance to sugar inhibition of both oxidases extracted from the commercial cultivar 'Elsanta' in comparison with the local cultivar 'Madame Moutot'. These results could be of importance in setting up the production processes of strawberry marmalades and jams, considering the high sugar content of such products.

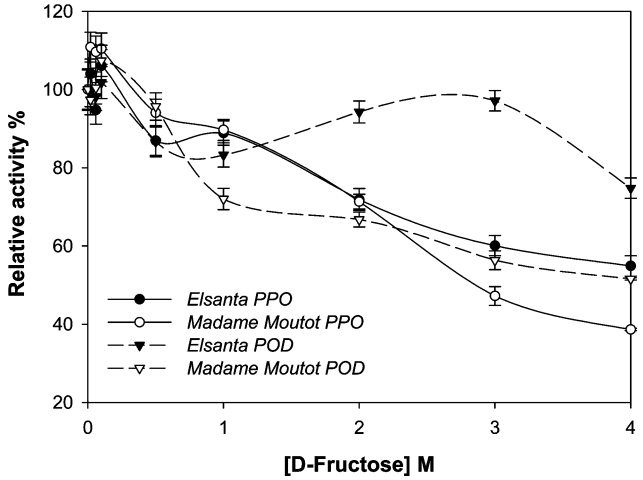

Figure 8. D-Fructose inhibition of crude strawberry PPO and POD.

Physicochemical Characteristics of Strawberry Cultivars. Samples from 'Elsanta' and 'Madame Moutot' strawberry cultivars were analyzed during 10 days of storage at 4 °C to find possible differences related to storage suitability of the two cultivars. Results are reported in **Table 2**. The percentages of titratable acidity (TA) in both cultivars were between 0.7 and 1.2% during the storage period. These values agree with those reported in the literature, between 0.6 and 2.3% (*31*, *32*). No significant changes in the citric acid content were observed during fruit storage at cool temperature, except for the 'Elsanta' cultivar, which showed a decrease of around 30% between days 0 and 3. Citric acid is the main compound accountable for titratable acidity, and it is predominant (over 90%) in strawberries. This acid regulates the cellular pH and may influence the anthocyanin stability and, as a consequence, the color of the fruit, although there is little published information about such a relationship (*33*). The initial value of total soluble solids (TSS) was 6.0 °Bx in both cultivars, significantly increasing throughout the storage period until reaching 8.4 °Bx in 'Elsanta' and 9.2 °Bx in 'Madame Moutot'. Considering that an acceptable strawberry flavor is achieved with a minimum TSS of 7.0 °Bx and a maximum TA of 0.8% (*34*), fruits from both cultivars reached the best quality for consumption after 3 days of cool storage. Dry matter percentage did not show significant variations during the storage of 'Madame Moutot', although a slight increase (from 7.6 to 8.5%) was seen probably due to evapotranspiration from the fruit surface. Such a phenomenon was more evident in 'Elsanta', leading to a significant increase of dry matter throughout the cool storage period. During the storage of strawberries at 4 °C, an anthocyanin content of cv. 'Madame Moutot' rose by about 43% from an initial 84.6 to 148.3 μg/g, whereas the anthocyanin content of cv. 'Elsanta' did not vary significantly throughout the storage period, although it did show an initial value (256.7 μg/g) 3 times higher than in 'Madame Moutot' and slightly increased up to the seventh day of storage. The increased pigmentation of fruits can be attributable to the activation of enzymes involved in phenylpropanoid metabolism induced by the low temperature according to other reports that found that cold storage stimulates anthocyanin biosynthesis in apples (*34*), pomegranates (*35*), lowbush blueberries (*36*), and red orange (*37*) fruits.

Strawberry Browning. Changes in L^*, a^*, b^*, chroma, and hue angle values were taken as a browning index of the samples

Table 2. Physicochemical Properties of 'Elsanta' and 'Madame Moutot' Strawberries during Storage at 4 °C[a]

cultivars	days at 4 °C	pH	TA (g of citric acid/100 g)	TSS (°Bx)	dry matter (%)	L^*	a^*	b^*	chroma	hue angle	anthocyanins (μg/g)
'Elsanta'	day 0	3.07 ± 0.06 b	1.23 ± 0.02 a	6.0 ± 0.3 b	8.1 ± 0.6 b	69.9 ± 4.1 a	41.1 ± 3.2 a	25.9 ± 1.4 bc	48.6	1.37	256.7 ± 67.1 a
	day 3	3.46 ± 0.06 a	0.84 ± 0.02 b	7.8 ± 0.4 a	9.2 ± 0.4 a	65.0 ± 3.0 a	36.7 ± 2.7 a	23.8 ± 2.0 c	43.8	1.32	351.3 ± 62.8 a
	day 7	3.47 ± 0.04 a	0.82 ± 0.04 b	8.4 ± 0.4 a	9.5 ± 0.6 a	57.2 ± 4.1 b	38.8 ± 3.5 a	28.2 ± 1.7 b	48.0	1.12	365.3 ± 51.4 a
	day 10	3.43 ± 0.05 a	0.86 ± 0.04 b	7.8 ± 0.5 a	9.0 ± 0.5 ab	44.7 ± 2.7 c	39.5 ± 3.6 a	34.0 ± 2.1 a	52.1	0.86	284.5 ± 60.4 a
'Madame Moutot'	day 0	3.42 ± 0.05 b	0.77 ± 0.07 a	6.0 ± 0.4 c	7.6 ± 0.8 a	74.2 ± 3.4 a	30.3 ± 2.9 a	30.2 ± 1.7 a	42.8	0.65	84.6 ± 18.3 b
	day 3	3.50 ± 0.04 a	0.75 ± 0.05 a	7.9 ± 0.3 b	7.9 ± 0.4 a	70.1 ± 3.8 ab	28.4 ± 3.5 a	29.3 ± 1.9 a	40.8	0.60	85.5 ± 16.9 b
	day 7	3.55 ± 0.03 a	0.84 ± 0.07 a	9.2 ± 0.4 a	8.0 ± 0.5 a	64.0 ± 2.6 bc	29.1 ± 2.4 a	29.5 ± 2.4 a	41.4	0.62	125.6 ± 21.4 ab
	day 10	3.55 ± 0.03 a	0.84 ± 0.03 a	9.2 ± 0.3 a	8.5 ± 0.6 a	61.0 ± 3.8 c	26.3 ± 1.8 a	26.1 ± 2.4 a	37.0	0.65	148.3 ± 30.3 a

[a] Means in the same column followed by the same letter are not significantly different at the $p \leq 0.05$ level according to Duncan's multiple range test.

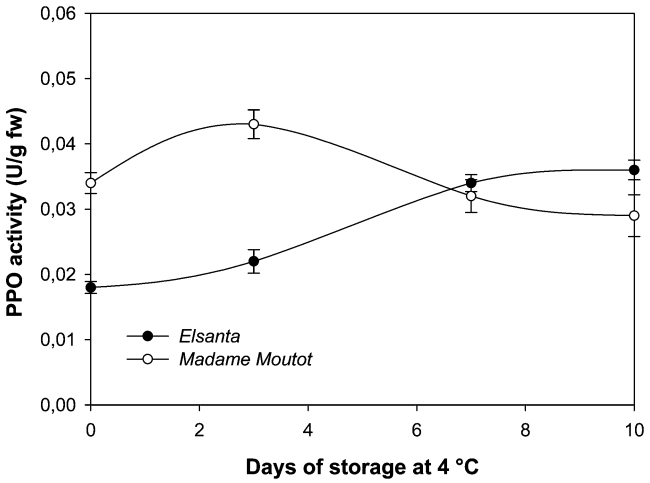

Figure 9. Activity of crude strawberry PPO during storage at 4 °C.

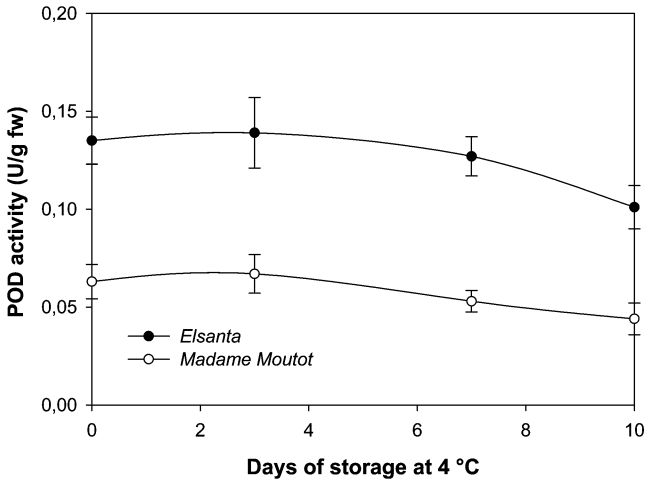

Figure 10. Activity of crude strawberry POD during storage at 4 °C.

during storage at 4 °C (**Table 2**). A decrease in L^* was evident in both cultivars but more marked in 'Elsanta'. In fact, the variation of lightness (ΔL^*) between days 0 and 10 was 25.2 and 13.2 for 'Elsanta' and 'Madame Moutot', respectively. Apart from a slight loss of brightness, 'Madame Moutot' strawberries did not show any other significant variation of color parameters, while a decrease of hue angle values (from an initial 1.37 to 0.86 at day 10) was also noticed in 'Elsanta'. Moreover, by calculating ΔE^* ($\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$), a widely used parameter for determining color differences perceptible by the human eye, it was possible to notice a sharp color variation during the storage period in both cultivars, but it was more marked in 'Elsanta' ($\Delta E^* = 26.5$ and 14.4 for 'Elsanta' and 'Madame Moutot', respectively). Degradation of color evidenced by ΔE^* was mostly due to dramatic depletion in lightness (L^*) values, whereas the green−red (a^*) and blue−yellow (b^*) chromatisms did not influence significantly such parameters. Furthermore, the changes in total anthocyanin amount of strawberries during cold storage did not seem to reflect such visible changes in juice color since increases of pH during storage probably influenced the color and stability of anthocyanins, as previously reported (*38*, *39*).

Polyphenol oxidase and peroxidase courses during cold storage are shown in **Figures 9** and **10**. PPO from cv. 'Madame Moutot' had a greater initial activity than 'Elsanta' (0.034 and 0.018 U/g fw, respectively) and manifested a maximum of 0.043 U/g fw on the third day of storage, while 'Elsanta' PPO increased throughout the storage period and showed maximum activity on the 10th day (0.036 U/g fw). On the other hand, POD was noticeably higher in 'Elsanta' during all storage period (0.135 and 0.063 U/g fw initial activity for 'Elsanta' and 'Madame Moutot', respectively); in both cases, the POD activity remained with little variations until the 10th day.

Table 3. Regression Coefficients (r^2) for Linear Regression Fitting of Browning Parameters

browning parameters		PPO	POD	L^*	a^*	b^*	chroma	hue angle
'Elsanta'	PPO		0.64	0.86	0.07	0.67	0.34	0.85
	POD	0.64		0.89	0.06	0.98	0.76	0.93
'Madame Moutot'	PPO		0.76	0.34	0.08	0.25	0.17	0.71
	POD	0.76		0.80	0.48	0.65	0.58	0.39

To establish the PPO and POD influence on strawberry browning, relationships between color measurements and enzymatic activities were researched, and regression coefficients r^2 for the linear regression fitting of these relations were calculated (**Table 3**). Results indicated that PPO and POD from cv. 'Elsanta' were very well-correlated with the parameter L^* ($r^2 = 0.86$ and 0.89, respectively) and hue angle ($r^2 = 0.85$ and 0.93, respectively). According to these results, the browning of the fruit seemed to be in relation to both PPO and POD activities. On the other hand, PPO was correlated only with the parameter L^* ($r^2 = 0.80$) in cv. 'Madame Moutot', whereas no clear correlation was found between POD activity and browning. The involvement of polyphenol oxidase in the browning of fruit and vegetables is well-known and reported in several studies about different products, such as bananas (*40*), peaches (*41*), and lettuce (*42*). In a previous study (*23*), we found a clear correlation between polyphenol oxidase and both browning and degradation of lycopene in cherry tomatoes. Besides, the role of peroxidase in enzymatic browning has been traditionally questioned mainly because of the low hydrogen peroxide content in fruit and vegetable tissues and the relatively high catalytic power of PPO for the phenolics. However, some authors (*10*, *43*) reported on the possible implication of peroxidase in enzymatic browning through a synergistic effect PPO−POD, through the generation of H_2O_2 in PPO-catalyzed reactions and

also the use by POD of semiquinonic intermediates of PPO-catalyzed reactions as oxidizing substrates. Results obtained confirmed the involvement of polyphenol oxidase also in the browning of cold stored strawberries, while only the commercial variety 'Elsanta' showed a good correlation between peroxidase activity and color loss of the samples.

ABBREVIATIONS USED

PPO, polyphenol oxidase; POD, peroxidase; TA, titratable acidity; TSS, total soluble solids; DOPAC, 3,4-dihydroxyphenylacetic acid; MBTH, 3-methyl-2-benzothiazolinone hydrazone; DMF, dimethylformamide.

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