Effect of pH on Pressure and Thermal Inactivation of Avocado Polyphenol Oxidase: A Kinetic Study

Carla A. Weemaes, Linda R. Ludikhuyze, Ilse Van den Broeck, and Marc E. Hendrickx*

Department of Food and Microbial Technology, Faculty of Agricultural and Applied Biological Sciences, Katholieke Universiteit Leuven, B-3001 Leuven, Belgium

High-pressure and thermal inactivations of avocado polyphenol oxidase (PPO) were studied for enzyme systems with pH 4−8. Both pressure and temperature inactivation of the enzyme followed first-order kinetics in the pH range 5−8 but showed deviation from simple first-order kinetics at pH 4. Threshold pressure and temperature for PPO inactivation, as well as pressure and temperature dependence of the inactivation rate constants, were influenced by pH. The minimum inactivation pressure increased from ∼450 MPa at pH 4 to ∼850 MPa at pH 8. The threshold temperature increased from ∼40 at pH 4 to ∼65 °C at pH 6 and was approximately constant at higher pH values. The z_p value and the absolute value of the activation volume, both reflecting the pressure dependency of the inactivation rate constant, respectively increased and decreased with increasing pH, in the pH range 5−8. The z_t value and activation energy, both reflecting the temperature dependency of the inactivation rate constant, respectively decreased and increased with increasing pH in the pH range 5−7. At pH 8, the activation energy was slightly lowered.

Keywords: *Polyphenol oxidase; thermal stability; pressure stability; pH; avocado (Persea americana Mill.)*

INTRODUCTION

Mechanical injury during postharvest storage or processing often causes browning of fruits and vegetables (Vámos-Vigyázó, 1981; McEvily et al., 1992). This enzymatic browning is caused by the release of polyphenol oxidase (PPO) from, for example, chloroplasts or microbodies, and the liberation of phenolic compounds from vacuoles, due to membrane disruption of these organelles. In the presence of oxygen, the enzyme catalyzes the conversion of *o*-diphenolic compounds into *o*-quinones, which subsequently polymerize nonenzymatically to brown pigments (Golan-Goldhirsh et al., 1984; Sapers, 1993). Apart from this color deterioration, enzymatic browning results in the development of off-flavors and a reduction of nutritional value (Vámos-Vigyázó, 1981; Golan-Goldhirsh et al., 1984; McEvily et al., 1992). The inhibition of enzymatic browning is therefore a great challenge in the food industry.

Avocados (*Persea americana* Mill.) are highly susceptible to enzymatic browning. A direct relationship between the browning susceptibility of avocado fruit and PPO activity was found by Kahn (1975). Later, Golan et al. (1977) reported that the browning rate of avocados is not only correlated with PPO activity but also with total phenol content of the fruit. No correlation with one specific phenolic compound (Golan et al., 1977) or with the carotenoid content of the avocado pear (Sharon-Raber and Kahn, 1983) was found.

Thermal processing is generally considered as the most effective method to inactivate PPO and, consequently, to inhibit enzymatic browning (Golan-Goldhirsh et al., 1984; McEvily et al., 1992). Thermal treatments can, however, be responsible for considerable sensorial and nutritional quality losses in fruits and vegetables (Lund, 1977; Sapers, 1993). In the case of avocados, it has for instance been reported that heating brings along the development of bitter off-flavors (Ben-et et al., 1973). The use of high hydrostatic pressure, on the other hand, is a new promising method to inactivate (spoilage) enzymes (Jolibert et al., 1994; Heinisch et al., 1995; Seyderhelm et al., 1996). This technique has furthermore been shown able to inactivate microorganisms (Butz and Ludwig, 1991; Shigehisa et al., 1991; Carlez et al., 1993; Raffalli et al., 1994) while leaving quality attributes (e.g., vitamins, flavors) intact (Kimura et al., 1994; Donsi et al., 1996; Yen and Lin, 1996). The quality retention of pressure-treated products is mostly ascribed to the inability of high pressure to break covalent bonds (Hayashi, 1989; Knorr, 1993). It should be mentioned, however, that some PPOs display activation, that is, enhancement of catalytic activity, upon pressurizing at "low" pressure (Asaka and Hayashi, 1991; Asaka et al., 1994; Anese et al., 1995). At higher pressures, a loss of PPO activity is observed (Gomes and Ledward, 1996).

Both pressure resistance and thermal resistance of enzymes are seriously dependent on environmental conditions such as pH (Curl and Jansen, 1950; Weemaes et al., 1997a) and the presence of sugars, salts, or additives (Jolibert et al., 1994; Ludikhuyze et al., 1996; Weemaes et al., 1997a). Compared to the influence of environmental conditions on thermal stability, the effect on pressure stability of food quality related enzymes is much less documented.

The aim of this study was to characterize the pressure and thermal stabilities of avocado PPO. Hereto, kinetic studies were performed to quantitatively describe the

* Author to whom correspondence should be addressed (telephone 32-16-32.15.85; fax 32-16-32.19.60; e-mail marc.hendrickx@agr.kuleuven.ac.be).

S0021-8561(97)00902-3 CCC: $15.00
Published on Web 06/06/1998

pressure and thermal inactivations of the enzyme. Such detailed, quantitative, kinetic studies are needed to implement new technologies (e.g., high-pressure processing) in the food industry. Moreover, the effect of the environmental factor pH on pressure and temperature resistance was determined. In this way, one will be able to select the most suitable environmental conditions to inactivate the enzyme by pressure or temperature.

MATERIALS AND METHODS

Extraction and Partial Purification of the Enzyme. Avocados were purchased at commercial maturity in a local supermarket. Avocado fruit (0.5 kg) was suspended in 0.5 L of distilled water, containing 2.5 mL of Triton-X-100 (Fluka Chemie, Buchs, Switzerland) and 5 g of insoluble PVP (Fluka Chemie). This suspension was mixed during 15 s (Waring commercial blender, New Hartford, CT) and filtered through four layers of cheesecloth. The filtrate was centrifuged (Beckman J2-HS centrifuge, Palo Alto, CA; 12000*g*, 4 °C, 15 min), and the resulting supernatant was subjected to ammonium sulfate (UCB, Drogenbos, Belgium) precipitation. The precipitate formed between 30 and 90% saturation was redissolved in distilled water and dialyzed overnight (membrane 6-27/32, Medicell International Ltd., London, England). The dialyzed suspension was lyophilized during 18 h (Christ Alpha 2-4 freeze-dryer, Osterode, Germany), and the resulting dry powder was stored in the freezer until the experimental setup.

Enzyme and Media. Partially purified and lyophilized avocado PPO was dissolved in 0.1 M phosphate buffer (pH 8−6) or in McIlvaine buffer (pH 6−4) at a concentration of 3 mg of lyophilized powder/mL of buffer. The pH values mentioned are those prevailing at atmospheric pressure. At elevated pressure, the pH value would be lower because of the negative ionization volume of phosphate buffer (Morild, 1981).

Activity Assay. The enzyme activity was measured spectrophotometrically (Biochrom 4060, UV−visible spectrophotometer, Pharmacia LKB Biochrom Ltd., Cambridge, England). Hereto, a certain amount of the 1−32-fold diluted enzyme extract was added to 1 mL of 0.01 M catechol solution [in phosphate buffer (0.1 M; pH 7)] so that the absorption increase (ΔOD/min) of the enzyme sample at inactivation time $t = 0$ maximally equaled 0.7. Up to this value, the relationship between enzyme concentration and enzyme activity was linear. The absorption increase, resulting from the oxidation of catechol, was measured at 393 nm and 23 °C. To calculate the enzyme activity (ΔOD/min) using linear regression, only the initial linear part of the absorption curve was taken into account.

Thermal Treatment. Kinetic parameter values (D, k, z_t, E_a) for thermal inactivation of avocado PPO were determined on the basis of isothermal inactivation experiments and calculation of the remaining activity of heat-treated samples. The enzyme samples contained in capillary tubes (Hirschmann, Eberstadt, Germany; 1.15 mm i.d., 150 mm length) were treated in a water bath with temperature control. After preset times, the samples were removed from the water bath and transferred to ice water to stop thermal inactivation instantaneously. After a storage period on ice water (t < 60 min), the remaining enzyme activity was measured. It was ascertained that no reactivation occurred during this storage period.

Pressure Treatment. Analogous to thermal inactivation, kinetic parameter values (D, k, z_p, V_a) for pressure inactivation of avocado PPO were derived on the basis of isobaric treatments and determination of the residual enzyme activity. Microcentrifuge tubes (Elkay, Hants, England; 250 μL) containing enzyme solution were pressurized during preset times in high-pressure equipment (HPIU-10.000 serial no. 95/1994, Resato, Roden, The Netherlands), with eight thermostated pressure vessels. The pressure transmitting fluid consisted of an oil-glycol mixture (TR15, Greenpoint Oil, Roden, The Netherlands).

In the case of pressure treatments, the initial activity (A_0) was not defined as the activity of a nontreated enzyme sample, as was done for thermal treatments, but as the activity of the sample when entering the time domain where pressure and temperature remained constant. This was done to exclude the variable pressure−temperature conditions resulting from pressure buildup and accompanied adiabatic heating, as was described in a previous paper (Weemaes et al., 1997a). This zero-point approach could be applied because of the first-order pressure inactivation behavior of avocado PPO.

After pressure release, the samples were stored on ice water (t < 60 min; no reactivation) and the enzyme activity was measured as described above.

Data Analysis. First-order kinetics (eq 1) are frequently reported for thermal and pressure inactivation of enzymes, in general (Svenson and Erikson, 1972; Seyderhelm et al., 1996; Ludikhuyze et al., 1997), and PPOs, in particular (Halim and Montgomery, 1978; Lee et al., 1983; Weemaes et al., 1997b). However, nth-order decay (eq 2), a two-step process (eq 3), or fractional conversion (eq 4) due to temperature or pressure processing is not exceptional (Galeazzi et al., 1981; Lourenço et al., 1990; Robert et al., 1995; Rizvi and Tong, 1997).

$$dA/dt = -kA \tag{1}$$

$$A = [A_0^{1-n} + (n-1)kt]^{1/(1-n)} \tag{2}$$

$$A = [A_1 - A_2k_1/(k_1 - k_2)]\exp(-k_1t) + [A_2(k_1/(k_1 - k_2))]\exp(-k_2t) \tag{3}$$

$$A = A_r + (A_0 - A_r)\exp(-kt) \tag{4}$$

The two-step model is based on a succession of two irreversible first-order steps, that is, an irreversible conversion of the native enzyme to an intermediate with lower specific activity and the subsequent irreversible conversion of the intermediate to an inactive enzyme form. Fractional conversion refers to a first-order inactivation process and takes into account the nonzero enzyme activity upon prolonged heating or pressurizing.

Next to characterization in terms of inactivation rate constants (k), first-order inactivations can be described by decimal reduction times, often referred to as D values. The D value is defined as the time (minutes) required, at a certain temperature, to reduce the initial enzyme activity (A_0) to $^1/_{10}$ of its original value and equals ln(10)/k. Pressure and temperature dependence of the D value can be expressed by z_p and z_t, respectively. These values represent the pressure or temperature increase needed to obtain a 10-fold reduction of D.

Next to z_p and z_t, pressure and temperature dependence of the inactivation rate constant can be expressed by the activation volume (V_a) and activation energy (E_a), respectively. The activation energy can be derived from the Arrhenius law (eq 5), whereas the activation volume can be derived from the Eyring equation (eq 6):

$$k = k_{ref}\exp\left[\left(\frac{-E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \tag{5}$$

$$k = k_{atm}\exp\left[\frac{-V_a(P - P_{ref})}{RT}\right] \tag{6}$$

To analyze the obtained data, the following procedure was used: a kinetic model describing the inactivation progress in time was at first selected. If the inactivation process could not adequately be described by a first-order kinetic model, the nth-order decay model, the two-isozyme model, the two-step model, and the fractional conversion model were critically considered to select the most suitable inactivation model.

Kinetic parameters for first-order inactivations were estimated using a two-step procedure: First, the natural or ten-based logarithms of the activity retentions (A/A_0) were

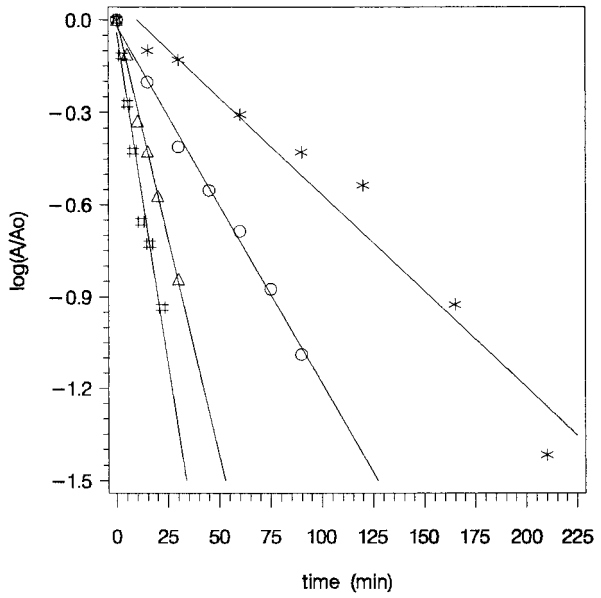

Figure 1. Pressure inactivation of avocado PPO (3 mg/mL; pH 5; 25 °C) at (*) 650, (○) 700, (△) 750, and (#) 800 MPa.

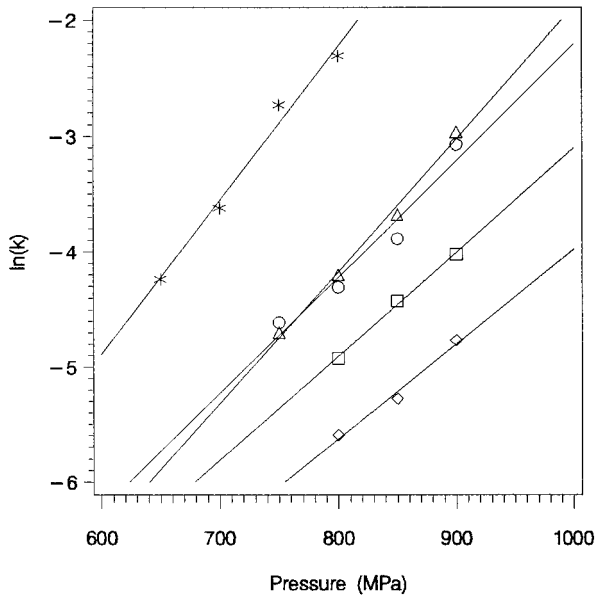

Figure 2. Variation with pressure of the inactivation rate constants: (*) pH 5, McIlvaine; (○) pH 6, McIlvaine; (△) pH 6, phosphate; (□) pH 7, phosphate; and (◇) pH 8, phosphate.

regressed versus treatment time (heating or pressurizing time) to obtain k or D values, respectively. Subsequently, the ten-based logarithms of the decimal reduction times were regressed versus pressure or temperature to calculate z_p or z_t, respectively. The activation volumes and activation energies were derived from the plots ln(k) versus P and ln(k) versus $1/T$, respectively.

In the case of non-log-linear decrease of enzyme activity, kinetic parameters (n and k for the nth-order decay model; A_1, A_2, k_1, and k_2 for the two-step model; A_L, A_S, k_L, and k_S for the two-isozyme model; A_r and k for the fractional conversion model) were estimated using nonlinear regression analysis on the inactivation data.

RESULTS AND DISCUSSION

Effect of pH on the High-Pressure Inactivation of Avocado PPO. The high-pressure inactivation of avocado PPO at room temperature (25 °C) was examined in the pH range 4−8. The minimum pressure needed to induce activity loss of avocado PPO in a reasonable time interval was found to be strongly dependent on the pH of the surrounding medium. Pressure inactivation in McIlvaine buffers with pH 4, 5, and 6 became apparent at, respectively, about 450, 650, and 750 MPa. When the enzyme was dissolved in phosphate buffer with pH 6 or 7, inactivation was noticeable upon pressurizing at about 750 and 800 MPa, respectively. At pH 6, the minimum pressure for PPO inactivation was not influenced by the type of buffer used (McIlvaine or phosphate buffer).

For enzyme systems dissolved in buffer at pH 5−8, pressure inactivation could be described by a first-order decay process. A typical example of such a first-order high-pressure inactivation process is given in Figure 1. The pressure dependence of the corresponding first-order inactivation rate constants is shown in Figure 2, from which it can readily be seen that the pressure resistance of avocado PPO decreases in the order pH 8 > pH 7 > pH 6 > pH 5. Activation volumes and z_p values, characterizing the pressure dependence of the inactivation rate constant, are summarized in Table 1. From this table it can be concluded that, in the pH range 5−8, z_p increases with increasing pH and that the absolute value of the activation volume decreases with increasing pH. The increase of z_p, or, conversely, the decrease of the absolute value of the activation volume with increasing pH, is almost linear ($r^2 = 0.94$ and 0.89, respectively). From Table 1 and Figure 2 it can furthermore be concluded that the activation volumes (or z_p values) for the pressure inactivation of avocado PPO in phosphate and McIlvaine buffer at pH 6 are not significantly different ($p > 0.05$).

Table 1. z_p and V_a Values for Pressure Inactivation of Avocado PPO in Buffers with Different pH Values

system	z_p (MPa)	V_a (cm^3/mol)
pH 4 (sensitive isozyme)	167.48 ± 18.70[a]	−34.08 ± 3.81[a]
pH 4 (resistant isozyme)	271.22 ± 24.37	−21.05 ± 1.90
pH 5; McIlvaine buffer	172.41 ± 16.00	−33.05 ± 3.01
pH 6; McIlvaine buffer	228.31 ± 37.27	−25.00 ± 4.10
pH 6; phosphate buffer	200.80 ± 12.54	−28.42 ± 1.78
pH 7; phosphate buffer	255.10 ± 16.46	−22.39 ± 1.44
pH 8; phosphate buffer	280.11 ± 37.11	−20.41 ± 2.70

[a] Standard error.

At pH 4, non-log-linear inactivation behavior was noticed when the enzyme was pressure-treated at 550−650 MPa. At lower (450−500 MPa) and higher (700−900 MPa) pressures an apparent first-order inactivation behavior was noticed. The non-log-linear inactivation curves at 550−650 MPa were consequently used to discriminate among the different non-first-order inactivation models. Since the enzyme preparation was previously found to consist of two isozymes (pI = 4.6 and 4.7; Weemaes et al., 1998), it was at first determined whether the "biphasic" curves were due to an overlap of two first-order inactivation processes with different inactivation rate constants; that is, it was determined whether the activity loss could be described by the summation of two exponential decays, one for the hypothesized labile isozyme and one for the hypothesized stable isozyme (eq 7):

$$A = A_L \exp(-k_L t) + A_S \exp(-k_S t) \qquad (7)$$

Bad parameter estimates (e.g., negative values for k_S) were, however, obtained. The model described by eq 7 was consequently discarded. The general nth-order

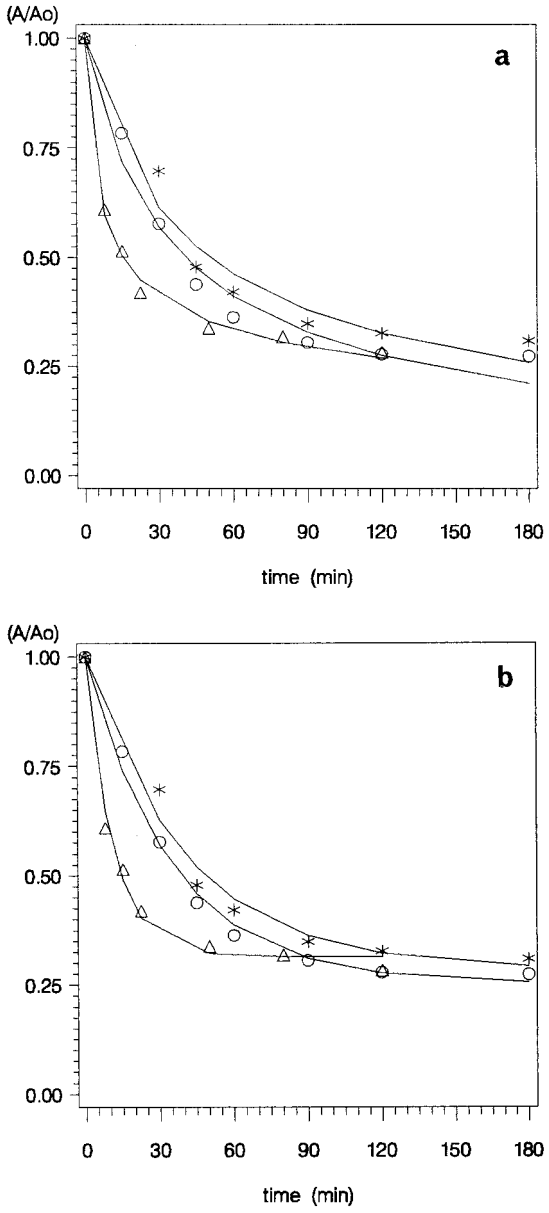

Figure 3. Pressure inactivation of avocado PPO (3 mg/mL; pH 4) at (*) 550, (○) 600, and (△) 650 MPa, modeled using (a) the general *n*th-order model and (b) the fractional conversion model.

model, the two-step model, and the fractional conversion model were consequently considered. In the case of the two-step model, the estimates for k_2 were again zero or slightly negative, so that there seemed to be no evidence for the occurrence of a subsequent, second inactivation step. The "fitting qualities" of the general *n*th-order model and the fractional conversion model are shown in Figure 3. Visual inspection of these inactivation curves revealed a misspecification of the general *n*th-order model at a high degree of inactivation. The fractional conversion model was therefore selected.

Taking into account all observations, that is, the observed first-order inactivation at low (450−500 MPa) and high (700−900 MPa) pressure and the fractional conversion behavior at intermediate pressures, it was suggested that the non-log-linear activity decrease was, after all, due to a large difference in pressure stability of the isozymes of avocado PPO at pH 4. The most pressure sensitive isozyme seemed to lose its activity upon pressurizing at ∼450 MPa, whereas the more

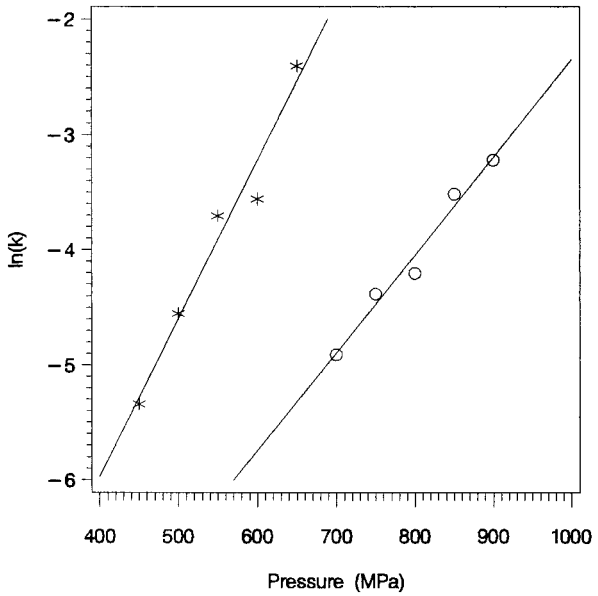

Figure 4. Variation with pressure of the inactivation rate constants for the pressure sensitive (*) and pressure resistant (○) isozyme of avocado PPO (3 mg/mL) at pH 4.

pressure resistant isozyme necessitated 700 MPa for inactivation. The apparent first-order inactivation at 450−500 MPa can thus be attributed to the first-order inactivation of the pressure labile isozyme, while the resistant isozyme is not affected by the pressure treatment. Because of the duration of the inactivation experiments at these pressures (2 h), no "activity plateau ($\sim A_r$)" was reached. The apparent first-order inactivation of avocado PPO at 700 MPa or higher pressures can, on the other hand, be ascribed to a complete inactivation of the pressure sensitive isozyme during the pressure buildup phase, which was excluded from the experiment because of its variable pressure−temperature conditions (see data analysis). The bad "fitting quality" of the two-isozyme model, described in eq 7, is most probably due to the fact that the most pressure resistant isozyme scarcely inactivated at the pressures at which the inactivation curves were recorded.

In the case of pressure inactivation of avocado PPO at pH 5−8, no evidence for a difference in pressure stability of the isozymes was found. In these conditions of pH, the PPO activity always decreased log-linearly as a function of pressurization time. It is therefore suggested that the isozymes only display distinct pressure stability at pH values <5. The reason for the arising difference in pressure stability of the isozymes when the pH was lowered to <5 is not known.

The inactivation curves obtained at 450−650 MPa were considered to calculate inactivation rate constants for the pressure sensitive isozyme, using the fractional conversion model. The inactivation data obtained at higher pressures were analyzed by assuming a first-order kinetic model.

The estimated inactivation rate constants for both isozymes are shown in Figure 4. The pressure sensitive isozyme at pH 4 seems to be more pressure sensitive than the enzyme population at pH 5. The pressure stability of the pressure resistant isozyme is on the same order of magnitude as the enzyme population at pH 6. The E_a values, estimated from Figure 4, and the corresponding z_p values are presented in Table 1. The

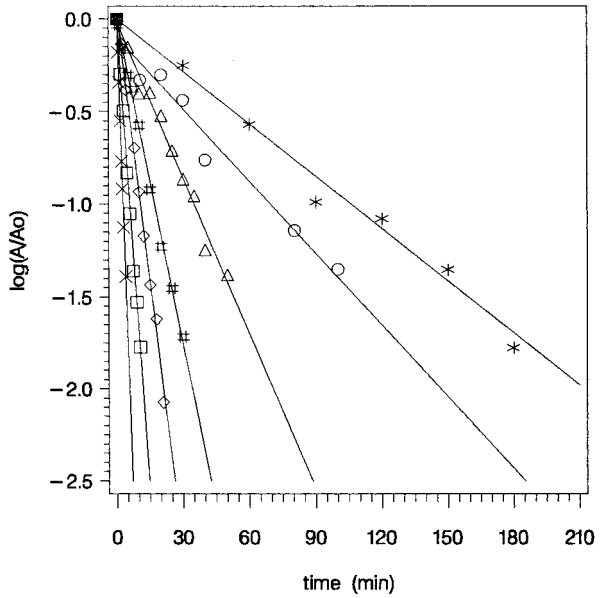

Figure 5. Thermal inactivation of avocado PPO (3 mg/mL; pH 6; McIlvaine buffer; atmospheric pressure) at (*) 62.5, (○) 65, (△) 67.5, (#) 70, (◇) 72.5, (□) 75, and (×) 77.5 °C.

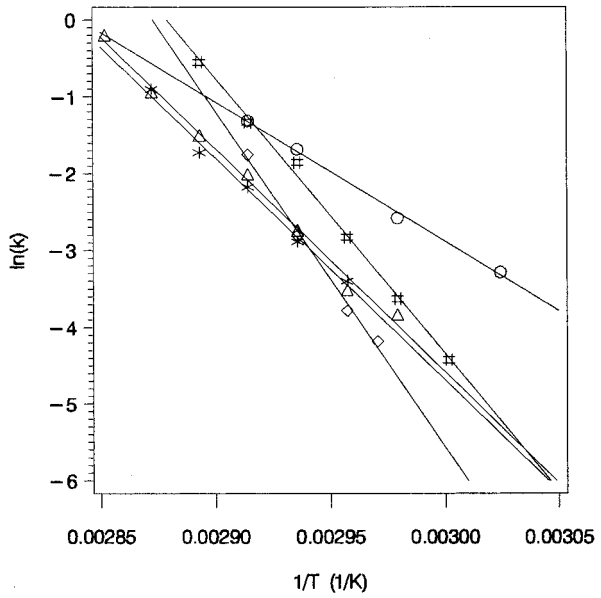

Figure 6. Variation with temperature of the inactivation rate constants: (○) pH 5, McIlvaine; (△) pH 6, McIlvaine; (*) pH 6, phosphate; (◇) pH 7, phosphate; and (#) pH 8, phosphate.

increase of the absolute value of the activation volume or, conversely, the decrease of z_p with decreasing pH, as was observed in the pH range 5−8, is continued only for the pressure sensitive isozyme at pH 4.

An analogous decrease in pressure resistance with decreasing pH has been noticed for mushroom PPO, although no change in inactivation behavior at low pH values was observed for this enzyme (Weemaes et al., 1997a). It might therefore be that an increasing pressure sensitivity with decreasing pH is a general characteristic of PPOs. However, more PPOs need to be screened to confirm this statement. The increased pressure sensitivity of the enzyme when dissolved in acid media is interesting with regard to potential applications since fruits and their derived products are mostly characterized by a low pH value and since acid products are the most likely candidates for high-pressure processing (Lechowich, 1993; Jolibert et al., 1994).

Effect of pH on the Thermal Inactivation of Avocado PPO. Thermal stability of avocado PPO at atmospheric pressure was determined for enzyme solutions with pH varying from 4 to 8. For avocado PPO solutions with pH 5−8, thermal inactivation could be adequately described by first-order kinetics, as was the case for pressure inactivation of the enzyme. As an example, the thermal inactivation of avocado PPO in McIlvaine buffer at pH 6 is presented in Figure 5. In the case of avocado PPO dissolved in buffer at pH 4, deviation from first-order inactivation kinetics was noted, as was the case for pressure inactivation of the enzyme at pH 4.

The minimum temperature needed to cause activity reduction of avocado PPO is greatly influenced by the environmental factor pH. At pH 4, 5, and 6 (McIlvaine buffer), thermal inactivation of the enzyme was noticeable at temperatures above about 40, 55, and 65 °C. In phosphate buffer (pH 6−8), temperatures above ∼60−65 °C were hereto needed. The threshold temperature for thermal inactivation of avocado PPO at pH 6−7 is in accordance with the value reported by Kahn (1977). In the case of mushroom PPO, a similar evolution of the minimum inactivation temperature with pH was noted: the minimum temperature for inactivation of mushroom PPO largely increased with pH in the pH range 4−6.5 and was about constant in the pH range 6.5−8 (Weemaes et al., 1997a). The minimum inactivation temperature at pH 6 was not influenced by the type of buffer used (phosphate or McIlvaine buffer).

Table 2. z_t and E_a Values for Thermal Inactivation of Avocado PPO in Buffers with Different pH Values

system	z_t (°C)	E_a (kJ/mol)
pH 4 (first step)	23.80 ± 3.24[a]	84.903 ± 12.501[a]
pH 4 (second step)	13.64 ± 2.63	147.773 ± 30.510
pH 5; McIlvaine buffer	14.39 ± 0.52	150.841 ± 6.161
pH 6; McIlvaine buffer	9.32 ± 0.32	241.705 ± 8.833
pH 6; phosphate buffer	9.37 ± 0.48	240.366 ± 12.960
pH 7; phosphate buffer	6.08 ± 0.32	363.546 ± 20.287
pH 8; phosphate buffer	7.27 ± 0.22	298.298 ± 9.025

[a] Standard error.

Figure 6 illustrates the temperature dependence of the inactivation rate constant k for the enzyme systems with pH 5−8. From this figure it is clear that temperature ranges have to be specified when thermal stabilities of the enzyme systems studied are compared. At temperatures <70 °C, the enzyme system at pH 5 is the most thermosensitive. At higher temperatures, the system at pH 8 is most temperature sensitive. The system at pH 6 is the most thermostable at temperatures >67 °C. At lower temperatures, the system at pH 7 is most thermostable. For each pH value, the corresponding activation energy (E_a) and z_t value for thermal inactivation are given in Table 2. From this table it is clear that the z_t and E_a values, respectively, decrease and increase with increasing pH up to pH 7. A further increase in pH to a value of 8 resulted in a slight increase of z_t and a slight decrease of E_a. It is furthermore clear from Table 2 and Figure 6 that the activation energy (or z_t value) for thermal inactivation at pH 6 is not influenced by the type of the buffer used ($p > 0.05$).

To find a mathematical model that could adequately describe the inactivation data obtained at pH 4, the appropriateness of the general nth-order model, two-isozyme model, two-step model, and fractional conver-

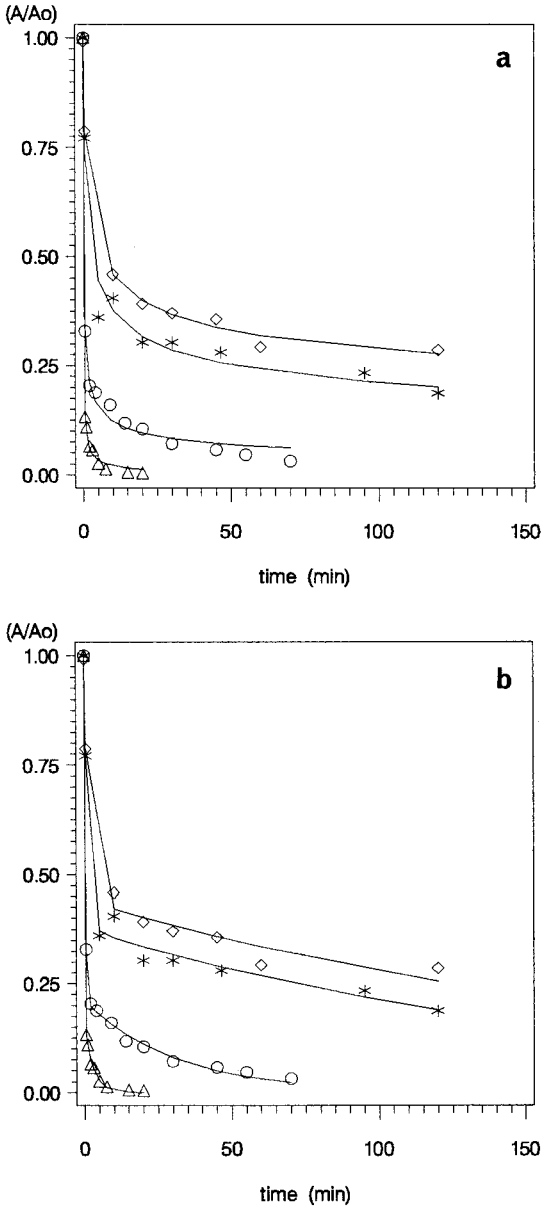

Figure 7. Thermal inactivation of avocado PPO (3 mg/mL; pH 4) at (◇) 40, (*) 45, (○) 57.5, and (△) 65 °C, modeled using (a) the general *n*th-order model and (b) the consecutive step model.

sion model was evaluated. In the case of the two-isozyme model, no convergence was obtained at the highest inactivation temperatures ($T \geq 57.5$ °C). The fractional conversion model also did not describe the inactivation data accurately, most probably because the enzyme activity continued to decrease upon prolonged temperature treatment; that is, there seemed to be no evidence for the presence of an extremely temperature resistant enzyme fraction. Since both the two-isozyme model and the fractional conversion model failed to describe the thermal inactivation data, it is clear that the deviation from first-order inactivation is not due to a difference in thermal stability of the two isozymes, when first-order kinetics for the latter are assumed.

Graphical comparison of the general *n*th-order model and the consecutive step model (see Figure 7) showed that these models could equally well describe the thermal inactivation data at pH 4. Because of the variation of the reaction order (n) with inactivation temperature (a linear decrease from 5.90 ± 0.21 at 40 °C to 2.45 ± 0.18 at 65 °C) and the high collinearity between the estimated parameters (n and k) when the inactivation data are analyzed by means of the general *n*th-order model, the consecutive step model was opted for to mathematically describe the thermal inactivation data of avocado PPO at pH 4. The inactivation rate constants for the two hypothesized consecutive steps are shown in Figure 8. The corresponding activation energies and z_t values are shown in Table 2. For all inactivation temperatures studied, the inactivation rate constants for both the first and second steps were higher than the inactivation rate constants determined for inactivation of avocado PPO at pH 5.

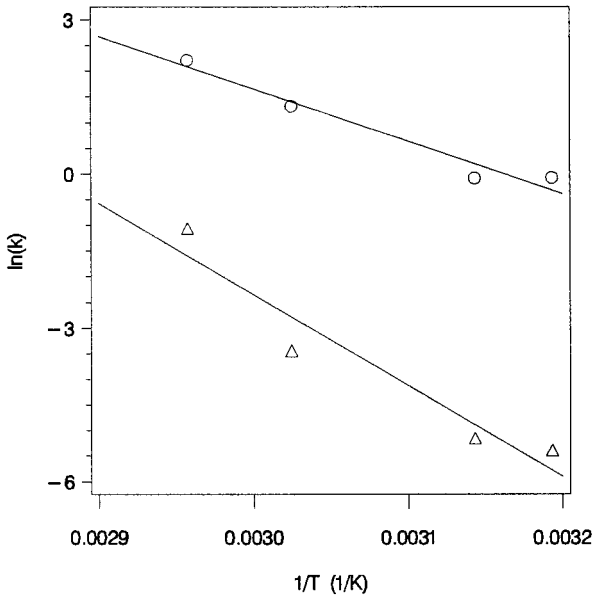

Figure 8. Variation with temperature of the inactivation rate constants of the (○) first and (△) second inactivation steps of avocado PPO (3 mg/mL) at pH 4.

McCord and Kilara (1983) observed an analogous two-step inactivation of mushroom PPO at low pH (pH 3.5). These authors hypothesized that the acidic pH resulted in a protonation of free carboxyl groups of the enzyme molecule, thereby neutralizing negative charges, thus promoting electrostatic repulsive forces due to positively charged amino groups. The remaining activity of the resulting partially disrupted tertiary structure of the enzyme might then be gradually lost due to the input of thermal energy. This hypothesis, however, holds only when heating of the buffer medium results in a decrease of the pH value. Since McIlvaine buffer is known to maintain constant pH upon heating, this explanation cannot account for the hypothesized two-step inactivation of avocado PPO.

An alternative explanation for the change in inactivation behavior when the pH of the enzyme solution was lowered to <5 was consequently looked for. It was hereto at first checked whether the sudden change in inactivation behavior was due to an altered interaction between citric acid (> McIlvaine buffer) and the enzyme, since the change in inactivation behavior occurred when the pK_a value of citric acid (4.76) was passed. Citric acid is known to act as a chelating agent upon the copper-containing PPO (McEvily et al., 1992; Sapers, 1993). Thermal inactivation of the enzyme in 0.2 M acetate buffer (pH 4 and 5) was hereto studied. Again, the inactivation at pH 5 followed first-order kinetics, whereas the inactivation at pH 4 displayed non-log-linear inactivation behavior. It could, therefore, be

concluded that the change in inactivation behavior is not due to an altered interaction with citric acid. A more general pH-dependent characteristic seems to be responsible for the change in inactivation mechanism. Possible explanations include a change in overall charge of the enzyme when the isoelectric point (4.6−4.7; Weemaes et al., 1998) is passed and a lowering of the strength of binding of copper at the active site. According to the former hypothesis, the enzyme shows first-order thermal inactivation kinetics when a net negative charge is prevailing (pH > p*I*) and deviation from first-order kinetic behavior when the overall charge is positive (pH < p*I*). No confirmation for this hypothesis was found in the literature. According to the latter hypothesis, the decreased strength of binding of the active site copper at pH 4 (Martinez and Whitaker, 1995) might influence the unfolding of the enzyme due to temperature processing.

When the effects of pH on thermal and pressure inactivation of avocado PPO are compared, it appears that in both cases the inactivation of avocado PPO can be affected in three ways: change of (i) inactivation behavior, (ii) threshold value of the denaturing agent (pressure/temperature), and (iii) dependence of the inactivation rate constant on the denaturing agent.

ABBREVIATIONS USED

OD, optical density; PPO, polyphenol oxidase; A, enzyme activity at time t (ΔOD/min); A_1, activity of the native enzyme (ΔOD/min); A_2, activity of the intermediate (ΔOD/min); A_L, activity of the native labile isozyme (ΔOD/min); A_0, enzyme activity (ΔOD/min) at time t = 0 or when entering the constant pressure−temperature phase; A_r, activity of the resistant enzyme fraction (ΔOD/min); A_S, activity of the native stable isozyme (ΔOD/min); D, decimal reduction time (min); E_a, activation energy (kJ/mol); k, inactivation rate constant [(response value)$^{1-n}$ × (min)$^{-1}$]; k_1, inactivation rate constant of the first reaction step [(response value)$^{1-n}$ × (min)$^{-1}$]; k_2, inactivation rate constant of the second reaction step [(response value)$^{1-n}$ × (min)$^{-1}$]; k_L, inactivation rate constant of the labile isozyme [(response value)$^{1-n}$ × (min)$^{-1}$]; k_S, inactivation rate constant of the stable isozyme [(response value)$^{1-n}$ × (min)$^{-1}$]; k_{atm}, inactivation rate constant at P_{ref} [(response value)$^{1-n}$ × (min)$^{-1}$]; k_{ref}, inactivation rate constant at T_{ref} [(response value)$^{1-n}$ × (min)$^{-1}$]; n, reaction order; P, pressure (MPa); P_{ref}, reference pressure (MPa); R, gas constant; t, time (min); T, temperature (K); T_{ref}, reference temperature (K); V_a, activation volume (cm^3/mol); z_p, z value for high-pressure processing (MPa); z_t, z value for thermal processing (°C).

LITERATURE CITED

Anese, M.; Nicoli, M. C.; Dall'aglio, G.; Lerici, C. R. Effect of high-pressure treatments on peroxidase and polyphenoloxidase activities. *J. Food Biochem*. **1995**, *18*, 285−293.

Asaka, M.; Hayashi, R. Activation of polyphenoloxidase in pear fruits by high-pressure treatment. *Agric. Biol. Chem*. **1991**, *55*, 2439−2440.

Asaka, M.; Aoyama, Y.; Ritsuko, N.; Hayashi, R. Purification of a latent form of polyphenoloxidase from La France pear fruit and its pressure activation. *Biosci., Biotechnol., Biochem*. **1994**, *58*, 1486−1489.

Ben-et, G.; Dolev, A.; Tatarsk, D. Compounds contributing to heat-induced bitter off-flavor in avocado. *J. Food Sci*. **1973**, *38*, 546−547.

Butz, P.; Ludwig, H. Hochdruckinaktivierung von Hefen und Schimmelpilzen (High-pressure inactivation of yeasts and molds). *Pharm. Ind*. **1991**, *53*, 584−586.

Carlez, A.; Rosec, J. P.; Richard, N.; Cheftel, J. C. High-pressure inactivation of *Citrobacter freundii*, *Pseudomonas fluorescens* and *Listeria innocua* in inoculated minced beef muscle. *Lebensm. Wiss. Technol*. **1993**, *26*, 357−363.

Curl, A. L.; Jansen, J. E. The effect of high pressures on pepsin and chymotrypsinogen. *J. Biol. Chem*. **1950**, *185*, 713−723.

Donsi, G.; Ferrari, G.; DiMatteo, M. High-pressure stabilization of orange juice: evaluation of the effects of process conditions. *Ital. J. Food Sci*. **1996**, *2*, 99−106.

Galeazzi, M. A. M.; Sgarbieri, V. C.; Constantinides, S. M. Isolation, purification and physicochemical characterization of polyphenoloxidase (PPO) from a dwarf variety of banana (*Musa cavendishii*, L.). *J. Food Sci*. **1981**, *45*, 150−155.

Golan, A.; Kahn, V.; Sodovski, A. Y. Relation between polyphenols and browning in avocado mesocarp. Comparison between the Fuerte and Lerman cultivars. *J. Agric. Food Chem*. **1977**, *25*, 1253−1260.

Golan-Goldhirsh, A.; Whitaker, J. R.; Kahn, V. Relation between structure of polyphenol oxidase and prevention of browning. *Adv. Exp. Med. Biol*. **1984**, *177*, 437−456.

Gomes, M. R. A.; Ledward, D. A. Effect of high-pressure treatments on the activity of some polyphenoloxidases. *Food Chem*. **1996**, *56*, 1−5.

Halim, D. H.; Montgomery, M. W. Polyphenoloxidase of d'Anjou pears (*Pyrus communis*, L.). *J. Food Sci*. **1978**, *43*, 603−605, 608.

Hayashi, R. Application of high pressure to food processing and preservation: philosophy and development. In *Engineering and Food*; Spiess, W. E. L., Schubert, H., Eds.; Elsevier Applied Science: London, England, 1989; pp 815−826.

Heinisch, O.; Kowalski, E.; Goossens, K.; Frank, J.; Heremans, K.; Ludwig, H.; Tauscher, B. Pressure effects on the stability of lipoxygenase; Fourier transform-infrared spectroscopy (FT-IR) and enzyme activity studies. *Z. Lebensm. Unters. Forsch*. **1995**, *201*, 562−565.

Jolibert, F.; Tonello, C.; Sagegh, P.; Raymond, J. Les effets des hautes pressions sur la polyphénoloxidase des fruits (Effect of high pressure on fruit polyphenol oxidase). *Bios Boissons Cond*. **1994**, *251*, 27−35.

Kahn, V. Polyphenol oxidase activity and browning of three avocado varieties. *J. Sci. Food Agric*. **1975**, *26*, 1319−1324.

Kahn, V. Some biochemical properties of polyphenoloxidase from two avocado varieties differing in their browning rates. *J. Food Sci*. **1977**, *42*, 38−43.

Kimura, K.; Ida, M.; Yoshida, Y.; Ohki, K.; Fukumoto, T.; Sakui, N. Comparison of keeping quality between pressure-processed jam and heat-processed jam: changes in flavor components, hue and nutrients during storage. *Biosci., Biotechnol., Biochem*. **1994**, *58*, 1386−1391.

Knorr, D. Effects of High-Hydrostatic-Pressure processes on food safety and quality. *Food Technol*. **1993**, *47*, 156−161.

Lechowich, R. V. Food safety implications of high hydrostatic pressure as a food processing method. *Food Technol*. **1993**, *47*, 170−172.

Lee, C. Y.; Smith, N. L.; Pennesi, A. P. Polyphenoloxidase from DeChaunac grapes. *J. Sci. Food Agric*. **1983**, *34*, 987−991.

Lourenço, E. J.; de Souza Leão, J.; Neves, V. A. Heat inactivation and kinetics of polyphenoloxidase from palmito (*Euterpe edulis*). *J. Sci. Food Agric*. **1990**, *52*, 249−259.

Ludikhuyze, L.; De Cordt, S.; Weemaes, C., Hendrickx, M.; Tobback, P. Kinetics for heat and pressure−temperature inactivation of *Bacillus subtilis* α-amylase. *Food Biotechnol*. **1996**, *10*, 105−129.

Ludikhuyze, L. R.; Van den Broeck, I.; Weemaes, C. A.; Herremans, C. H.; Van Impe, J. F.; Hendrickx, M. E.; Tobback, P. P. Kinetics for isobaric−isothermal inactivation of *Bacillus subtilis* α-amylase. *Biotechnol. Prog*. **1997**, *13*, 532−538.

Lund, D. B. Design of thermal processes for maximizing nutrient retention. *Food Technol*. **1977**, *31*, 71−78.

Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

McCord, J. D.; Kilara, A. Control of enzymatic browning in processed mushrooms (*Agaricus bisporus*). *J. Food Sci.* **1983**, *48*, 1479−1483.

McEvily, A. J.; Iyengar, R.; Otwell, W. S. Inhibition of enzymatic browning in foods and beverages. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 253−273.

Morild, E. The theory of pressure effects on enzymes. *Adv. Protein Chem.* **1981**, *34*, 93−166.

Raffalli, J.; Rosec, J. P.; Carlez, A.; Dumay, E.; Richard, N.; Cheftel, J. C. Stress et inactivation par haute pression de *Listeria innocua* introduites dans une crème laitière (Stress and high-pressure inactivation of *Listeria innocua* introduced into dairy cream). *Sci. Aliment.* **1994**, *14*, 349−358.

Rizvi, A. F.; Tong, C. H. Fractional conversion for determining texture degradation kinetics of vegetables. *J. Food Sci.* **1997**, *62*, 1−7.

Robert, C. M.; Cadet, F. R.; Rouch, C. C.; Pabion, M.; Richard-Forget, F. Kinetic study of the irreversible thermal deactivation of palmito (*Acanthophoenix rubra*) polyphenol oxidase and effect of pH. *J. Agric. Food Chem.* **1995**, *43*, 1143−1150.

Sapers, G. M. Browning of foods: control by sulfites, antioxidants and other means. *Food Technol.* **1993**, *47*, 75−84.

Seyderhelm, I.; Boguslawski, S.; Michaelis, G.; Knorr, D. Pressure induced inactivation of selected food enzymes. *J. Food Sci.* **1996**, *61*, 308−310.

Sharon-Raber, O.; Kahn, V. Avocado mesocarp; browning potential, carotenoid content, polyphenol oxidase, catalase and peroxidase activities: comparison between six avocado cultivars. *J. Food Sci.* **1983**, *48*, 1874−1875.

Shigehisa, T.; Ohmori, T.; Saito, A.; Taji, S.; Hayashi, R. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *Int. J. Food Microbiol.* **1991**, *12*, 207−216.

Svenson, S. G.; Erikson, C. E. Thermal inactivation of lipoxygenase from peas (*Pisum sativum* L.). Time−temperature relationships and pH-dependence. *Lebensm. Wiss. Technol.* **1972**, *5*, 118−123.

Vámos-Vigyázó, L. Polyphenoloxidase and peroxidase in fruits and vegetables. *Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49−127.

Weemaes, C. A.; De Cordt, S. V.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E.; Tobback, P. P. Influence of pH, benzoic acid, EDTA, and Glutathione on the pressure and/or temperature inactivation kinetics of mushroom polyphenoloxidase. *Biotechnol. Progr.* **1997a**, *13*, 25−32.

Weemaes, C.; Rubens, P.; De Cordt, S.; Ludikhuyze, L.; Van den Broeck, I.; Hendrickx, M.; Heremans, K.; Tobback, P. Temperature sensitivity and pressure resistance of mushroom polyphenoloxidase. *J. Food Sci.* **1997b**, *62*, 261−266.

Weemaes, C. A.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E.; Tobback, P. P. Activity, electrophoretic characteristics and heat inactivation of polyphenoloxidases from apples, avocados, grapes, pears and plums. *Lebensm. Wiss. Technol.* **1998**, *31*, 44−49.

Yen, G. C.; Lin, H. T. Comparison of high-pressure treatment and thermal pasteurization effects on the quality and shelf-life of guava puree. *Int. J. Food Sci. Technol.* **1996**, *31*, 205−213.

Received for review October 21, 1997. Revised manuscript received April 20, 1998. Accepted April 29, 1998. This research has been supported by the Flemish Institute for the Promotion of Scientific-Technological Research in Industry (IWT), the KULeuven Research Council (Project OT/94/19), the National Fund for Scientific Research (Project G.0189.95), and the European Commission (Project FAIR-CT96-1175).

JF970902S