Inactivation of Orange Pectinesterase by Combined High-Pressure and -Temperature Treatments: A Kinetic Study

Ilse Van den Broeck, Linda R. Ludikhuyze, Ann M. Van Loey, and Marc E. Hendrickx*

Laboratory of Food Technology, Department of Food and Microbial Technology, Faculty of Agricultural and Applied Biological Sciences, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium

Pressure and/or temperature inactivation of orange pectinesterase (PE) was investigated. Thermal inactivation showed a biphasic behavior, indicating the presence of labile and stable fractions of the enzyme. In a first part, the inactivation of the labile fraction was studied in detail. The combined pressure−temperature inactivation of the labile fraction was studied in the pressure range 0.1−900 MPa combined with temperatures from 15 to 65 °C. Inactivation in the pressure−temperature domain specified could be accurately described by a first-order fractional conversion model, estimating the inactivation rate constant of the labile fraction and the remaining activity of the stable fraction. Pressure and temperature dependence of the inactivation rate constants of the labile fraction was quantified using the Eyring and Arrhenius relations, respectively. By replacing in the latter equation the pressure-dependent parameters (E_a, k_{refT}) by mathematical expressions, a global model was formulated. This mathematical model could accurately predict the inactivation rate constant of the labile fraction of orange PE as a function of pressure and temperature. In a second part, the stable fraction was studied in more detail. The stable fraction inactivated at temperatures exceeding 75 °C. Acidification (pH 3.7) enhanced thermal inactivation of the stable fraction, whereas addition of Ca^{2+} ions (1 M) suppressed inactivation. At elevated pressure (up to 900 MPa), an antagonistic effect of pressure and temperature on the inactivation of the stable fraction was observed. The antagonistic effect was more pronounced in the presence of a 1 M $CaCl_2$ solution as compared to the inactivation in water, whereas it was less pronounced for the inactivation in acid medium.

Keywords: *Orange pectinesterase; thermal stability; pressure stability; kinetics; stable fraction*

INTRODUCTION

One of the main problems in the fruit juice industry is the maintenance of the turbidity of juices. It is generally accepted that cloud loss is initiated by the enzymatic reaction of pectinesterase (PE), which demethylates pectin. The low-methoxy pectin formed may be cross-linked by divalent cations, leading to precipitation of the pectins and hence to juice clarification. The fact that sensory properties such as flavor, color, texture, and aroma of, for example, citrus juices are partly attributable to cloud (Klavons et al., 1994) explains why consumers expect a stable cloud in most citrus juices and citrus-based beverages and perceive its presence as an indication of quality.

Thermal treatment (e.g., 1 min at 90 °C) is a conventionally used method to inactivate PE (Eagerman and Rouse, 1976). It is suggested that PE should be used as a target in the establishment of the process because the thermal resistance of PE is higher than that of the relevant spoiling microorganisms (Nath and Ranganna, 1977; Massaguer et al., 1994). However, because the thermal treatment is accompanied by undesired quality losses, research into alternatives is stimulated.

Inhibition of PE by polygalacturonic acid or pectic acid hydrolysates (Alonso et al., 1997; Termote et al., 1977) or by an inhibitor purified from kiwi (Balestrieri et al., 1990; Castaldo et al., 1991) has been reported. Flocculation of the cloud components can also be prevented by the addition of ammonium oxalate, which removes the bivalent cation (Krop and Pilnik, 1974).

In recent years, a number of studies have been conducted on the application of high pressure as a substitute for thermal treatment for inactivation of PE and preservation of citrus fruit juices and have already showed some promise (Ogawa et al., 1990; Takahashi et al., 1993; Parish et al., 1994; Parish, 1998; Irwe and Olsson, 1994; Goodner et al., 1998). As high pressure affects noncovalent bonds and does not accelerate most chemical changes, flavor and appearance are usually superior to those of comparable products preserved by heat (Aleman et al., 1994). However, some enzymes (Seyderhelm et al., 1996; Quaglia et al., 1996; Weemaes et al., 1998; Hendrickx et al., 1998) seem to be very pressure stable. Therefore, high-pressure treatments will most likely be accompanied by other treatments, such as mild heating (Farr, 1990). By a combination of high pressure and moderate temperature elevation microbial safe products can be produced (Gould, 1973; Butz et al., 1990; Seyderhelm and Knorr, 1992).

Most of the studies related to the inactivation of orange PE by pressure are qualitative. Therefore, the objective of this work was to study the combined pressure−temperature inactivation of orange PE on a kinetic basis. Commercially available as well as self-extracted orange PE was considered.

* 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).

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MATERIALS AND METHODS

Extraction and Partial Purification of Orange PE. Navel oranges (from Spain) were purchased from a local supermarket in December 1997. The extract was prepared according to a modification of the method of Körner et al. (1980). Oranges were peeled and squeezed with the aid of a juice centrifuge. The juice was discarded, and the pulp (segment covers and juice sacs), which contains the largest amount of PE (Rombouts et al., 1982), was collected. All treatments were carried out at 4 °C. Orange pulp was homogenized in a 1 M NaCl solution for 1 min by a blender. The ratio of orange material to extractant was 1:1. Crude PE was extracted by stirring the homogenate at pH 7.0 for 2 h. The pH was maintained by the addition of 0.01 N NaOH. Afterward, the homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged (3200*g*; 4 °C; 15 min), and the obtained supernatant was subjected to ammonium sulfate (UCB, Drogenbos, Belgium) precipitation. The fraction precipitating between 30 and 80% saturation was retained and redissolved in 10 mM, pH 5.0, acetate buffer. The enzyme solution was further dialyzed four times against a 10 mM, pH 5.0, acetate buffer solution. The precipitate obtained during the dialysis was removed by centrifugation at 1200*g*. Finally, the extract was lyophilized for 18 h (Christ Alpha 2-4 freeze-dryer, Osterode, Germany) and kept in a freezer until further use.

Enzyme Solution. Pectinesterase purified from orange peel [EC 3.1.1.11, Sigma (Bornem, Belgium), product P-5400) (PE)] was purchased as a lyophilized powder containing 270 units/mg of solid. One unit was defined by Sigma as the release of 1 μequiv of acid from pectin within 1 min at a pH of 7.5 and a temperature of 30 °C. Orange PE dissolved in deionized water was used as a model system (pH 4.5). The influence of pH and Ca^{2+} ions was investigated by inactivating PE in a citric acid buffer, 5 mM, pH 3.7, and a 1 M $CaCl_2$ solution, respectively. To study the combined pressure–temperature inactivation of the labile fraction of orange PE under more realistic conditions, PE was extracted from oranges (see Extraction and Partial Purification of Orange PE) and dissolved in a citric acid buffer 5 mM, pH 3.7, a pH relevant for orange products. A citric acid buffer was chosen because of the relatively low negative ionization volume of this buffer (Kitamura and Itoh, 1987) and because of the occurrence of citric acid in natural products such as fruits and vegetables. A low molarity of the buffer was required because the activity of PE was determined titrimetrically (see Activity Assay).

No prior equilibrium dialysis was conducted to exchange the enzyme solution with citric acid buffer or calcium chloride.

Activity Assay. PE activity was determined titrimetrically at a pH of 7.0 and a temperature of 22 °C. The reaction mixture in the standard assay method consisted of 250 μL of PE sample and 30 mL of a 0.35% apple pectin solution [70–75% esterification, supplied by Fluka (Bornem, Belgium)] containing 0.125 M NaCl. Before injection of the enzyme solution, the pectin solution was adjusted to pH 7.0. During hydrolysis at 22 °C, the pH was maintained at 7.0 by the addition of 0.01 N NaOH using an automatic pH-stat titrator (Methrom). Every 15 s the consumption of 0.01 N NaOH was recorded during the 10 min reaction period. The PE activity is proportional to the consumption rate of NaOH ($\Delta V_{NaOH}/\Delta t$).

Isothermal Treatment. Isothermal inactivation experiments were performed in a water bath with temperature control. To ensure isothermal heating, the enzyme solution was enclosed in capillary tubes (Hirschmann, 1.15 mm i.d., 150 mm length) using a vacuum pump. After preset time intervals, the capillaries were withdrawn from the water bath and immediately cooled in ice water to stop thermal inactivation. The residual enzyme activity was measured after 10–120 min of storage in ice water. It was verified experimentally that during 24 h of storage the enzyme that was partially inactivated by temperature did not reactivate. The temperature range studied varied from 60 to 82 °C. The blank (A_0) was defined as the activity of a non-heat-treated enzyme sample.

Isobaric–Isothermal Treatment. To perform isobaric–isothermal treatments, a laboratory-scale, multivessel high-pressure instrument (HPIU-10.000 serial no. 95/1994, Resato, Roden, The Netherlands) was used. The apparatus allows pressurization up to 1000 MPa in combination with temperatures ranging from −20 to 100 °C. High pressure is generated using a pressure intensifier in the central pressure circuit. The pressure medium is a glycol–oil mixture (TR15, Resato). A thermostated mantle, which surrounds each vessel and which is connected to a cryostat, controls the temperature. This apparatus is suited for kinetic studies, since eight individual vessels (volume = 8 mL, diameter = 10 mm, length = 100 mm) can be subjected to the same pressure level and the same temperature level.

Isobaric–isothermal treatments were performed as follows: flexible microcentrifuge tubes (0.3 mL, Elkay) filled with solution to be pressurized were enclosed in the pressure vessels, already equilibrated at a preset temperature. Pressure was built up slowly to minimize adiabatic heating. After the desired pressure had been reached, the individual vessels were isolated so that the pressure was maintained in the vessels until the valves were opened. On the basis of previous research (Weemaes et al., 1997), an equilibration period of 1–2 min to allow temperature to evolve to its desired value (input value) was taken into account. By starting the time course of the experiment ("zero point") after this equilibration period, the process could be considered as an isobaric–isothermal treatment. At this moment, one pressure vessel was decompressed and the activity of the corresponding enzyme sample was considered as the blank (A_0). The other seven vessels, each containing one enzyme sample, were then decompressed after preset time intervals.

After pressure release, the samples were immediately cooled in ice water. The residual activity was measured titrimetrically after 10–120 min of storage in ice water. It was again verified experimentally that the enzyme that was partially inactivated by pressure did not reactivate during 24 h of storage. The pressure range studied varied from 50 to 900 MPa in combination with temperatures ranging from 15 to 82 °C.

Data Analysis. *First-Order Model.* Inactivation of enzymes can often be described by a first-order kinetic model that is, enzyme activity decreases log-linearly as a function of time (see eq 1), where A_t is the enzyme activity at time t, A_0 is initial

$$\ln(A_t/A_0) = -kt \tag{1}$$

enzyme activity, t is treatment time, and k is the first-order inactivation rate constant. This relation is valid under isothermal and isothermal–isobaric conditions.

Biphasic Model. Enzymes characterized by several isoenzymes, such as orange PE (Versteeg et al., 1980), can often be grouped into two fractions, one more thermal resistant than the other and both inactivating according to a first-order decay kinetic model. If it is assumed that the inactivation of both fractions is independent of one another, the inactivation can be modeled according to eq 2 (Chen and Wu, 1998), where α

$$A_t/A_0 = \alpha \exp(-k_s t) + (1 - \alpha) \exp(-k_l t) \tag{2}$$

is the fraction of thermostable enzyme, $(1 - \alpha)$ is the fraction of thermolabile enzyme, k is the first-order inactivation rate constant, and the subscript s and l for k denote thermostable and thermolabile, respectively. This relation is valid at a constant temperature and/or pressure.

First-Order Fractional Conversion Model. A fractional conversion model is a special case of a first-order model. Fractional conversion f takes into account a nonzero activity after prolonged heating and/or pressurizing ($=A_\infty$) and can be expressed mathematically as

$$f = \frac{A_0 - A_t}{A_0 - A_\infty} \tag{3}$$

For most irreversible first-order reactions, A_∞ approaches 0 and eq 3 can be reduced to the following:

$$f = \frac{A_0 - A_t}{A_0} \quad (4)$$

A plot of the logarithm of $(1 - f)$ versus time yields a straight line with a rate constant expressed by the negative of the slope (Levenspiel, 1972).

$$\ln(A_t/A_0) = \ln(1 - f) = -kt \quad (5)$$

So, it is clear that eq 5 is identical to eq 1 when A_∞ approaches 0.

To account for the nonzero activity after prolonged heating and/or pressurizing, fractional conversion in the following form should be used:

$$\ln(1 - f) = \ln\left(\frac{A_t - A_\infty}{A_0 - A_\infty}\right) = -kt \quad (6)$$

Rearranging eq 6 results in eq 7. By plotting A_t (activity after different time intervals) versus time, the inactivation rate constant of the labile fraction (k value) and the remaining activity after treatment (A_∞ value) can be estimated using nonlinear regression:

$$A_t = A_\infty + (A_0 - A_\infty)\exp(-kt) \quad (7)$$

This relationship is valid in the temperature and/or pressure domain where only the labile fraction inactivates, whereas the activity of the stable fraction (A_∞ value) does not change with respect to time. This nonzero activity may or may not be a function of applied temperature and pressure.

Temperature and Pressure Dependence of the Inactivation Rate Constant. Once the k values are estimated by one of the models described above, its temperature and pressure dependence can be derived. The temperature dependence of k is given by the activation energy (E_a), as indicated in the Arrhenius relationship. Equation 8 gives the linearized form of this relationship:

$$\ln k = \ln k_{ref} + \left[\frac{E_a}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (8)$$

The activation energy can be obtained through linear regression when the logarithm of the rate constant is plotted versus the inverse of temperature. This relation is valid at constant pressure.

The pressure dependence of k is expressed by the activation volume ($\Delta V^{\ddagger}$), as presented in eq 9 in linearized form (Eyring equation), and can be obtained through linear regression when the logarithm of the rate constant is plotted versus pressure. This relationship is valid at constant temperature.

$$\ln k = \ln k_{atm} - \left[\frac{\Delta V^{\ddagger}}{R_p T}(P - P_{ref})\right] \quad (9)$$

RESULTS AND DISCUSSION

Thermal Inactivation of Orange PE. Thermal inactivation of commercially available orange peel PE (1 mg of solid/mL of deionized water) was studied in the temperature domain 60−82 °C. Two different fractions were observed: a heat labile fraction and a heat stable fraction. A biphasic model was used to estimate the kinetic inactivation parameters (see Table 1). For temperatures up to 70 °C, the inactivation rate constant of the labile fraction was estimated, whereas the stable fraction did not inactivate and could be considered as a constant fraction. Five percent of the initial activity was estimated to be thermostable. At higher temperatures (75−82 °C), the inactivation rate constant of the stable fraction was estimated. In this temperature domain, the labile fraction inactivated too quickly to estimate accurately the decline of activity. On the basis of the inactivation rate constants of the labile and stable fractions, the activation energy was calculated. The inactivation rate constant of the labile fraction was found to be less temperature sensitive than the inactivation rate constant of the stable fraction. This confirms literature data (Wicker and Temelli, 1988; Versteeg et al., 1980).

Table 1. Kinetic Parameters for Isothermal Inactivation at Atmospheric Pressure of Commercial Orange Peel PE (1 mg/mL) in Water

	biphasic model	fractional conversion
Inactivation Rate Constant of the Heat Labile Fraction (min^{-1})		
60 °C	0.110 ± 0.013[a]	0.103 ± 0.004[a]
63 °C	0.311 ± 0.014[a]	0.314 ± 0.008[a]
65 °C	0.610 ± 0.106[a]	0.637 ± 0.064[a]
67 °C	1.896 ± 0.128[a]	1.766 ± 0.095[a]
70 °C	4.189 ± 0.491[a]	3.848 ± 0.376[a]
% heat stable PE	5.3 ± 0.6[a]	5.5 ± 0.6[a]
E_a of the heat labile fraction (kJ/mol)	357.4 ± 21.7[b]	353.1 ± 17.7[b]
Inactivation Rate Constant of the Heat Stable Fraction (min^{-1})		
75 °C	0.037 ± 0.004[a]	
77 °C	0.112 ± 0.021[a]	
80 °C	0.551 ± 0.041[a]	
82 °C	1.234 ± 0.473[a]	
E_a of the heat stable fraction (kJ/mol)	518.2 ± 19.3[b]	

[a] Asymptotic standard error of regression ($n \approx 10$). [b] Standard error of regression ($n = 5$ or 4).

In the temperature domain 60−70 °C, where only the heat labile fraction inactivated, also a fractional conversion model was used to estimate the inactivation parameters (see Table 1). The inactivation parameters estimated by both models, that is, the inactivation rate constant of the thermolabile fraction, the percentage of thermostable PE and the activation energy for the labile fraction correspond very well.

Also, other heat inactivation studies indicated the presence of fractions of PE with differing heat stabilities, whereby the residual activity observed at 70 °C was not destroyed until temperatures around 90 °C were applied (Rothschild et al., 1975; Wicker and Temelli, 1988). Versteeg et al. (1980) reported the thermal inactivation of PE to be biphasic and hypothesized that the two phases were due to two typical pseudo-first-order inactivation reactions involving reacting mixtures of two fractions of differing thermal sensitivities.

Thermal Inactivation of the Stable Fraction of Orange PE. As mentioned in the previous paragraph, 5% of the initial activity of orange peel PE was found to be thermostable. Also in other fruits such as white and red grapefruit (Seymour et al., 1991a,b; Cameron and Grohmann, 1995), tomatoes (Laratta et al., 1995), kiwi fruit (Giovane et al.,1990), green beans (Laats et al., 1997), cherries (Alonso et al., 1996), and mandarin oranges (Rillo et al., 1992), different PE isoforms with different thermostabilities were detected. The amount of thermostable PE might vary with geographic location, growth practice, postharvest handling, seasonal differences, cultivars (Snir et al., 1996), fruit tissues (Cameron et al., 1997), and experimental differences in protocol (Wicker, 1992). The presence of thermostable PE, even if the amount is small, can destabilize cloud stability (Versteeg et al., 1980). Therefore, this fraction should be inactivated. We investigated the influence of

Table 2. Kinetic Parameters for Isothermal Inactivation at Atmospheric Pressure of the Stable Fraction of Commercial Orange Peel PE in the Presence and Absence of Ca^{2+} Ions (1 M) and at Acid pH (Citric Acid Buffer, pH 3.7)

	absence of Ca^{2+} ions	presence of Ca^{2+} ions	buffer pH 3.7
Inactivation Rate Constant of the Heat Stable Fraction (min^{-1})			
65 °C			0.006 ± 0.001^a
67 °C			0.011 ± 0.001^a
68.5 °C			0.029 ± 0.005^a
70 °C			0.101 ± 0.019^a
73 °C		0.008 ± 0.001^a	0.367 ± 0.024^a
75 °C	0.037 ± 0.004^a		
77 °C	0.112 ± 0.021^a	0.046 ± 0.003^a	
80 °C	0.551 ± 0.041^a	0.221 ± 0.022^a	
82 °C	1.234 ± 0.473^a	0.471 ± 0.056^a	
E_a of the Heat Stable Fraction (kJ/mol)			
	518.2 ± 19.3^b	472.9 ± 14.8^b	533.1 ± 42.6^b

[a] Asymptotic standard error of regression ($n \approx 10$). [b] Standard error of regression ($n = 4$ or 5).

pH and Ca^{2+} ions as sensitizing factors on the inactivation of the stable fraction of commercial orange peel PE (1 mg/mL). Temperatures ranged from 65 to 82 °C. The influence of pH on the isothermal inactivation of the thermostable fraction of orange peel PE was investigated by using a 5 mM citric acid buffer at the pH of commercially available orange juice, that is, pH 3.7. Because of the low molarity of the citric acid buffer (5 mM), the pH of the buffer solution during thermal treatment was measured and confirmed to be constant. To investigate the influence of Ca^{2+} ions on the thermal inactivation of the thermostable fraction of orange peel PE, the enzyme was dissolved in a 1 M $CaCl_2$ solution. An overview of the kinetic parameters, estimated using a biphasic model, is presented in Table 2. Treatment in a citric acid buffer, pH 3.7, accelerated thermal inactivation of the stable fraction as compared to the inactivation in water (pH 4.5). The activation energy remained similar. Also, the thermolabile fraction was found to be less thermostable in an acid medium (Van den Broeck et al., 1999). The presence of Ca^{2+} ions (1 M), on the other hand, clearly reduced the inactivation rate of the thermostable fraction of orange peel PE. The activation energy of the stable fraction was only slightly influenced by the addition of Ca^{2+} ions. This is in contrast with the results obtained for the labile fraction, for which a decreased thermal stability and a decreased temperature sensitivity of the k value were noted (Van den Broeck et al., 1999).

Combined Pressure–Temperature Inactivation of the Labile Fraction of Orange PE. The pressure–temperature inactivation of commercially available orange peel PE (0.4 mg/mL) was investigated in deionized water, whereas the inactivation of self-extracted orange PE (2 mg/mL) was studied in citric acid buffer, 5 mM, pH 3.7. The latter experiments were performed to approach more realistic conditions. Pressures from 0.1 to 900 MPa were applied in combination with temperatures from 15 to 65 °C. The experimental domain of combined pressure–temperature inactivation of orange PE had to be demarcated due to limitations in the experimental design. The duration of the experiments carried out was chosen between 20 and 220 min, depending on the conditions of pressure and temperature. At all temperatures and/or pressures studied no complete inactivation of orange PE was attained. Only the labile fraction inactivated, whereas the activity of the stable fraction remained unchanged with respect to time. The fractional conversion model was used to fit the inactivation data. In Figure 1 the inactivation of commercial orange peel PE is illustrated for different combinations of pressure and temperature. The remaining active fraction after treatment varied between 3 and 8% of the initial activity, irrespective of the applied pressure and/or temperature. This implies that a similar reduction could be obtained whether PE was inactivated by temperature or pressure, suggesting that the remaining activity after pressure treatment represented heat stable PE.

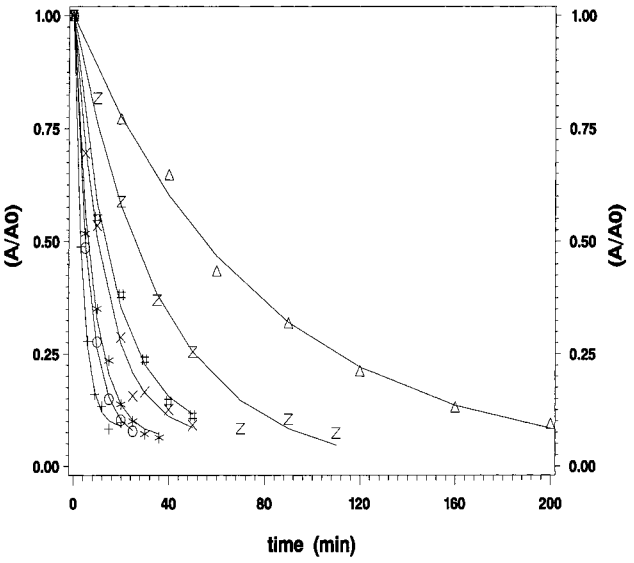

Figure 1. Combined pressure–temperature inactivation of commercial orange peel PE at the following pressure–temperature combinations (MPa/°C): (#) 0.1/57; (○) 0.1/60; (Δ) 100/57; (Z) 150/60; (×) 400/60; (+) 800/35; (∗) 850/20.

The estimated inactivation rate constants of the labile fraction of commercial and self-extracted orange PE for several combinations of constant pressure and temperature are summarized in, respectively, Tables 3 and 4. A comparison of both tables reveals that self-extracted orange PE in citric acid buffer, pH 3.7, inactivates more rapidly at a given pressure–temperature combination than commercially available orange peel PE in deionized water. However, comparing both tables one should take into account that both the origin of the orange PE (commercial citrus PE is prepared from the citrus peel and may not have the same ratio of isoenzymes as found in the self-extracted orange PE from the pulp) and the environmental conditions differ. It has previously been reported that commercial orange peel PE is less pressure stable in citric acid buffer (pH 3.7) than in water (pH 4.5) (Van den Broeck et al., 1999). For both sources of orange PE a pressure increase to 700–900 MPa at room temperature was required to inactivate the labile fraction. At higher temperatures the inactivation rate accelerated, indicating the synergistic effect of pressure and temperature. In the high-temperature ($T \geq 57$ °C) and low-pressure ($P \leq 300$ MPa) region, on the contrary, a clearly antagonistic effect of pressure and temperature was observed. Increasing pressure caused a decrease of the inactivation rate constant. Information about the secundary, tertiary, and quaternary structures of orange PE might be useful to explain the observed antagonistic effect of pressure and tempature. However, up to now, this information is lacking. A similar antagonistic effect

Table 3. *k* Values ($\times 10^2$ min $^{-1}$) for Combined Pressure−Temperature Inactivation of the Labile Fraction of Commercial Orange Peel PE in Deionized Water

P (MPa)	*T* (°C)											
	20	25	30	35	40	45	50	55	57	60	63	65
0.1									5.90 ± 0.51[a]	15.34 ± 0.47	50.70 ± 2.63	94.20 ± 2.57
50									2.69 ± 0.13	5.44 ± 0.56	19.54 ± 1.49	
100									1.28 ± 0.09	3.37 ± 0.13	10.76 ± 1.16	
150									1.21 ± 0.07	2.71 ± 0.29	8.95 ± 1.75	
200									1.47 ± 0.09	2.92 ± 0.18	9.16 ± 2.37	
250									1.33 ± 0.17	2.39 ± 0.31		
300									1.59 ± 0.10	3.72 ± 1.51	6.43 ± 0.89	
350									4.58 ± 1.00	4.88 ± 0.70		
400									6.50 ± 0.91	7.49 ± 0.57	21.21 ± 1.65	
450							1.11 ± 0.12	3.02 ± 0.23	6.64 ± 0.51	7.78 ± 1.24		
500							3.23 ± 0.25	5.82 ± 0.44	8.60 ± 0.38			
550						1.84 ± 0.77	4.88 ± 1.07	9.54 ± 0.88	10.17 ± 0.98			
600					3.23 ± 0.30	5.18 ± 0.45	8.24 ± 0.38	24.20 ± 0.92				
650				3.99 ± 0.15	6.45 ± 0.35	9.69 ± 0.48						
700				7.63 ± 0.63	10.55 ± 0.50	14.28 ± 0.43	22.92 ± 0.78					
750	3.56 ± 0.35	7.15 ±0.17	7.24 ± 0.25	14.88 ± 0.28	17.08 ± 0.35							
775			9.85 ± 0.35									
800	5.55 ± 0.15	11.89 ± 0.27	10.70 ± 0.34	26.79 ± 0.94								
825			18.82 ± 1.09									
850	12.57 ± 0.85	24.08 ± 1.59		46.96 ± 0.55								
900	18.93 ± 1.64	29.47 ± 1.40										

[a] Standard error of regression ($n = 8$).

Table 4. *k* Values ($\times 10^2$ min $^{-1}$) for Combined Pressure−Temperature Inactivation of the Labile Fraction of Self-Extracted Orange PE in Citric Acid Buffer, pH 3.7

P (MPa)	*T* (°C)												
	15	20	25	30	35	40	45	50	55	57	60	63	65
0.1									8.27 ± 1.42[a]	13.27 ± 2.23	21.32 ± 3.09	45.13 ± 6.39	102.74 ± 14.08
100									2.65 ± 0.12				
200									1.57 ± 0.21				
300									2.07 ± 0.31				
350								0.85 ± 0.13					
400								1.36 ± 0.09	3.30 ± 0.29				
450						0.82 ± 0.16		1.89 ± 0.32	5.97 ± 1.13				
500						1.15 ± 0.09	1.38 ± 0.14	4.13 ± 0.12					
550				1.50 ± 0.15	1.67 ± 0.17	2.19 ± 0.15	2.92 ± 0.30						
600	1.39 ± 0.04	1.97 ± 0.21	3.95 ± 0.26	3.19 ± 0.17	3.40 ± 0.14	4.36 ± 0.55	6.76 ± 0.13						
650	3.13 ± 0.13	3.50 ± 0.17	4.66 ± 0.48	5.21 ± 0.38	5.28 ± 0.58	6.92 ± 1.22	8.96 ± 0.41						
700	6.79 ± 0.69	5.38 ± 0.22	5.41 ± 0.19	6.66 ± 2.08	14.29 ± 0.57	21.27 ± 0.18	20.80 ± 3.63						
750	11.94 ± 0.63	7.92 ± 1.31	11.94 ± 0.63	10.61 ± 0.80									
800		15.31 ± 1.23											

[a] Standard error of regression ($n = 8$).

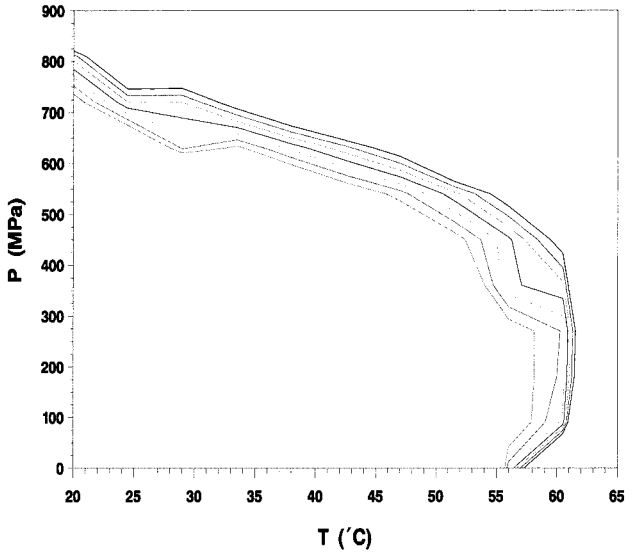

Figure 2. Pressure–temperature kinetic diagram for the *P*/*T* inactivation of commercial orange peel PE in deionized water. The inner and outer lines represent *P*/*T* combinations for which $k = 0.02$ and 0.08 min^{-1}, respectively.

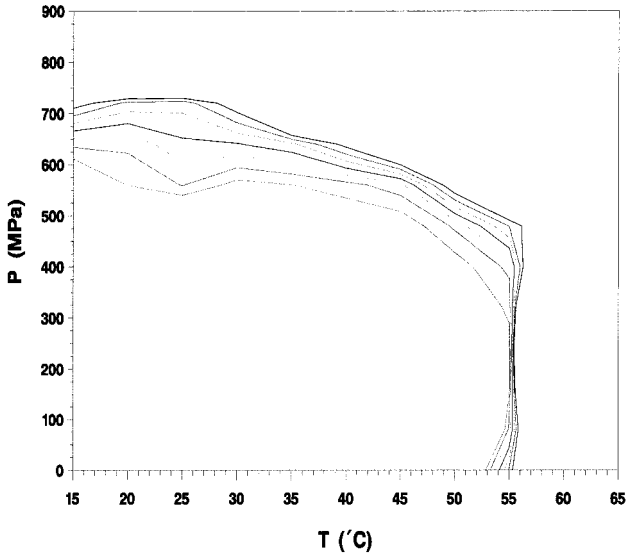

Figure 3. Pressure–temperature kinetic diagram for the *P*/*T* inactivation of self-extracted orange PE in citric acid buffer. The inner and outer lines represent *P*/*T* combinations for which $k = 0.02$ and 0.08 min^{-1}, respectively.

was observed for polyphenol oxidase (Weemaes et al., 1998).

On the basis of the estimated *k* values from isothermal as well as isobaric–isothermal inactivation experiments (see Tables 3 and 4), a pressure–temperature kinetic diagram (isorate contour) for commercial orange peel PE (see Figure 2) and self-extracted orange PE (see Figure 3) was constructed. The lines in this two-dimensional diagram represent combinations of pressure and temperature, resulting in the same inactivation rate constant of the labile fraction. The synergistic effect of pressure and temperature in the high-pressure region ($P \geq 350$ MPa) and the antagonistic effect of pressure and temperature in the low-pressure–high-temperature domain can be deduced from the shape of this kinetic diagram.

In a preliminary step to define a mathematical model describing the combined effect of temperature and pressure on the inactivation rate constant *k*, the temperature dependence (see eq 8) and pressure dependence (see eq 9) of *k* were evaluated. Because of the observed antagonistic effect of pressure and temperature, the Eyring relation, expressing the pressure dependence of the inactivation rate constant at a constant temperature, was not valid in the entire pressure domain. Therefore, for estimation of the activation volume ($\Delta V^{\ddagger}$), as illustrated in Figure 4, the pressure area was restricted to pressures >350 MPa for commercial orange peel PE and >200 MPa for self-extracted orange PE. The values for the activation volumes are presented in Table 5. From this table, it can be derived that the inactivation rate constant is clearly less pressure sensitive at temperatures at which at atmospheric pressure inactivation occurs, that is, 55 and 57 °C for, respectively, self-extracted orange PE in citric acid buffer pH 3.7 and commercial orange peel PE in water. Comparison of the inactivation in water and in acid medium showed a decreased pressure sensitivity of the inactivation rate constant for the inactivation in acid medium in the lower (20–30 °C) and higher temperature domains (45–55 °C). Also, Basak and Ramaswamy (1996) found a decreased pressure sensitivity of the inactivation rate constant at lower pH values.

The Arrhenius relation, in contrary to the Eyring relation, was valid in the whole pressure domain studied; that is, the inactivation rate could be enhanced

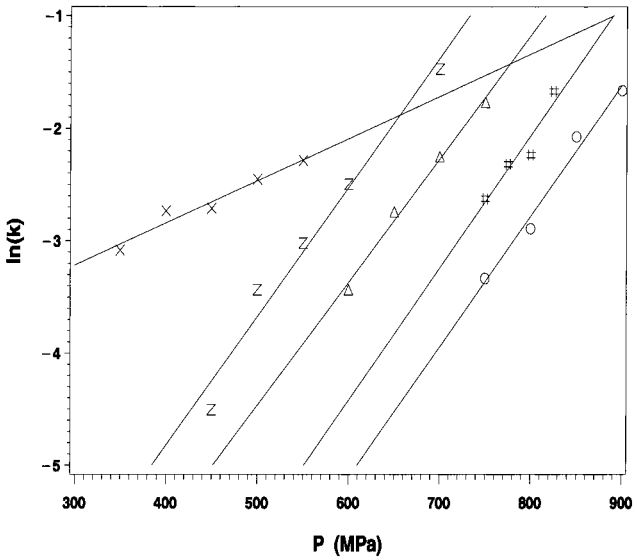

Figure 4. Pressure dependence of the *k* values at different temperatures: (○) 20; (#) 30; (Δ) 40; (Z) 50; (×) 57 °C.

Table 5. $\Delta V^{\ddagger}$ Values for Pressure Inactivation of Commercial Orange Peel PE (in Deionized Water) and Self-Extracted Orange PE (in Citric Acid Buffer, pH 3.7) at Atmospheric and Elevated Temperatures

	$\Delta V^{\ddagger}$ (cm^3/mol)	
T (°C)	commercial PE in deionized water	self-extracted PE in citric acid buffer
15		−34.57 ± 1.96
20	−28.39 ± 2.71[a]	−23.95 ± 1.23
25	−24.55 ± 3.44	−17.18 ± 5.42
30	−29.72 ± 6.50	−23.43 ± 2.51
35	−31.68 ± 0.67	−35.25 ± 3.76
40	−28.58 ± 1.86	−33.22 ± 2.64
45	−35.81 ± 5.44	−34.64 ± 2.68
50	−30.90 ± 3.02	−27.22 ± 3.48
55	−31.34 ± 2.50	−13.77 ± 2.92
57	−10.29 ± 1.35	

[a] Standard error of regression ($n = 4–6$).

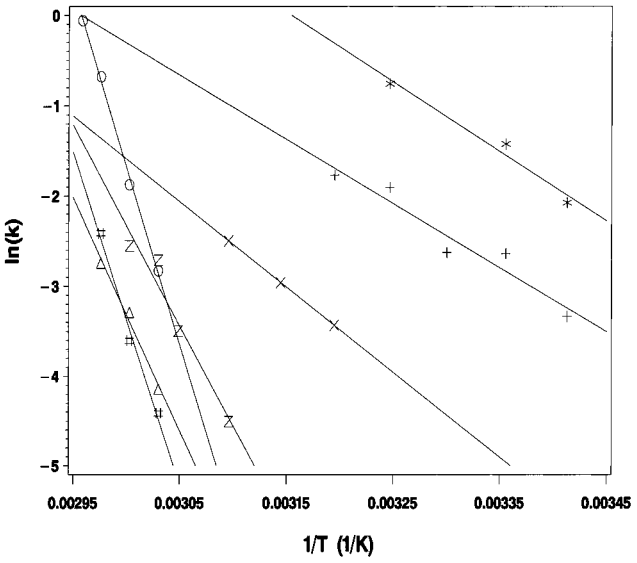

Figure 5. Temperature dependence of the k values at different pressures: (○) 0.1; (#) 150; (Δ) 300; (Z) 450; (×) 600; (+) 750; (∗) 850 MPa.

by increasing temperature at a constant pressure (see Tables 3 and 4). By plotting the logarithm of the inactivation rate constant as a function of reciprocal temperature at constant pressure (see Figure 5), activation energies at different pressure levels were determined by linear regression. The estimated activation energies are presented in Table 6. It can be derived that, for commercial orange peel PE, the activation energy is ∼300−325 kJ/mol in the low-pressure domain. With increasing pressure a linear decrease of the activation energy was observed. In the high-pressure domain a value of ∼60−70 kJ/mol was estimated. A similar behavior was observed for the activation energy of self-extracted orange PE. However, the values of the activation energy for the inactivation of self-extracted orange PE in buffer are lower than those observed for the inactivation in water. The relationship between the activation energy (E_a) and pressure (P) could be simplified by a linear equation (eq 10). The parameter estimates are presented in Tables 7 and 8 for commercial and self-extracted orange PE, respectively.

$$E_a = f - eP \tag{10}$$

Literature data confirm that orange PE can be only partly inactivated by pressure treatment, indicating the existence of a pressure stable fraction (Parish et al., 1994; Ogawa et al., 1992; Cano et al., 1997). Basak and Ramaswamy (1996) hypothesized the pressure inactivation of citrus PE (P < 400 MPa) to be biphasic. However, they ascribed the biphasic behavior to the dual effect of the pressure treatment; the first one is an instantaneous pressure kill (IPK), which depends only on the pressure level, and the second one depends on both pressure level and holding time. Only for the latter effect could the first-order rate kinetics be applied. Also, Goodner et al. (1998) described pressure inactivation of orange PE (P > 500 MPa) by a biphasic model. They ascribed the initial drop in activity to an inactivation of the heat labile form of PE, whereas the remaining activity illustrated the effect of pressure on the heat stable form. However, only for pressures of 500 and 600 MPa was a first-order inactivation rate constant of the labile fraction reported. Pressures of 700 MPa and higher inactivated the heat labile form too quickly to allow measurement of the decrease of activity. A longer processing time did not indicate any inactivation of the remaining heat stable form, indicating that the heat stable form is also pressure resistant. Seyderhelm et al. (1996) reported the effect of higher pressure on PE, but the data given were for commercial PE in pH 7 Tris buffer at 45 °C. The shortest processing time shown, 2 min, was sufficient to completely inactivate PE at 900 MPa.

Table 6. E_a Values for Thermal Inactivation of Commercial Orange Peel PE (in Deionized Water) and Self-Extracted Orange PE (in Citric Acid Buffer) at Atmospheric and Elevated Pressure

	E_a value (kJ/mol)	
P (MPa)	commercial PE in deionized water	self-extracted PE in citric acid buffer
0.1	326.956 ± 11.669[a]	220.30 ± 21.43
50	304.475 ± 52.550	
100	326.923 ± 18.873	
150	307.128 ± 35.842	
200	281.138 ± 41.728	
300	215.208 ± 26.087	
400	181.445 ± 80.653	
450	184.994 ± 29.872	106.24 ± 32.10
500	120.112 ± 15.906	107.33 ± 45.44
550	127.030 ± 15.212	36.36 ± 5.18
600	78.769 ± 0.313	33.70 ± 6.56
650	72.209 ± 2.718	25.19 ± 2.35
700	59.496 ± 5.030	38.11 ± 9.56
750	59.052 ± 9.278	
800	69.234 ± 20.545	
850	63.965 ± 10.976	

[a] Standard error of regression (n = 3−7).

Formulation of a Mathematical Model To Predict the Inactivation of the Labile Fraction. On the basis of the inactivation data, a model that describes the dependence of the inactivation rate constant of the labile fraction on pressure and temperature was formulated. Recently, kinetic models describing pressure−temperature dependence of the inactivation rates of *Bacillus subtilis* α-amylase, avocado polyphenol oxidase, and soybean lipoxygenase have been reported (Ludikhuyze et al., 1997, 1998; Weemaes et al., 1998). From these case studies, the inactivation kinetics of polyphenol oxidase is very similar to the inactivation kinetics of orange PE. Therefore, a similar approach in developing a mathematical model was followed. The Arrhenius equation was used as the starting point, because this relation was valid at all pressures studied. The two pressure-dependent parameters in the Arrhenius equation, namely, E_a and k_{ref}, were both replaced by a mathematical expression reflecting their pressure dependency. The pressure dependence of E_a was already presented in eq 10. The pressure dependence of k_{ref} (T_{ref} = 333 K) could be described by a third-degree polynomial model (eq 11). This is visualized in Figure 6 for

$$\ln k_{ref} = a + bP + cP^2 + dP^3 \tag{11}$$

commercial orange peel PE. Parameter estimates for a, b, c, and d are presented in Tables 7 and 8 for, respectively, commercial and self-extracted orange PE.

Substitution of E_a and k_{ref} by eq 10 and 11 transforms the Arrhenius equation (eq 8) into eq 12.

$$\ln k = a + bP + cP^2 + dP^3 + \left[\frac{f - eP}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{12}$$

Table 7. Parameter Estimates for Pressure and/or Temperature Inactivation of Commercial Orange Peel PE

	estimated value		
parameter	eq 9	eq 10	eq 11
e	0.370 ± 0.022[a]		0.348 ± 0.027
f	336.82 ± 11.57		325.12 ± 18.55
a		-1.91 ± 0.11	-1.88 ± 0.10
b		$-(22.42 \pm 2.22) \times 10^{-3}$	$-(17.55 \pm 1.09) \times 10^{-3}$
c		$(84.32 \pm 11.87) \times 10^{-6}$	$(53.27 \pm 3.26) \times 10^{-6}$
d		$-(83.17 \pm 17.31) \times 10^{-9}$	$-(35.95 \pm 2.79) \times 10^{-9}$

[a] Asymptotic standard error of regression ($n = 64$).

Table 8. Parameter Estimates for Pressure and/or Temperature Inactivation of Self-Extracted Orange PE

	estimated value		
parameter	eq 9	eq 10	eq 11
e	0.290 ± 0.039[a]		0.248 ± 0.036
f	223.91 ± 20.77		193.44 ± 22.89
a		-2.49 ± 0.09	-2.39 ± 0.17
b		$-(15.36 \pm 2.11) \times 10^{-3}$	$-(19.00 \pm 1.83) \times 10^{-3}$
c		$(39.86 \pm 11.84) \times 10^{-6}$	$(55.20 \pm 5.94) \times 10^{-6}$
d		$-(16.76 \pm 17.43) \times 10^{-9}$	$-(38.50 \pm 5.35) \times 10^{-9}$

[a] Asymptotic standard error of regression ($n = 47$).

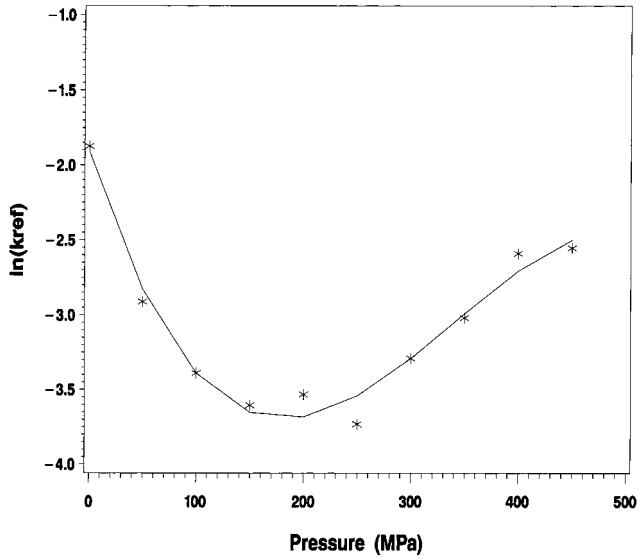

Figure 6. Pressure dependence of ln k_{ref} of commercial orange peel PE at 60 °C.

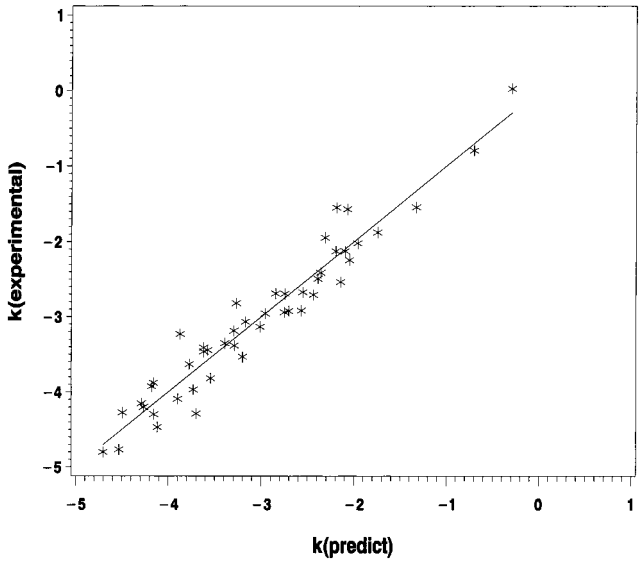

Figure 7. Correlation between the *k* values of self-extracted orange PE, determined from experimental work, and the *k* values estimated using the model described in eq 12.

The values of the kinetic parameters characterizing this model are shown in Tables 7 (commercial orange peel PE) and 8 (self-extracted orange PE). Using this model (eq 12), the inactivation data of commercial orange peel PE in water as well as self-extracted orange PE in citric acid buffer pH 3.7 could be accurately fit. To analyze the quality of parameter estimation, a plot of experimental *k* values versus *k* values calculated using the estimated parameters was made. This is illustrated in Figure 7 for the inactivation of self-extracted orange PE. A similar plot was obtained for commercial orange peel PE. The divergence from the bisector can be seen as an indicator for the accuracy of the model and its parameters; that is, the more the calculated and estimated values mutually differ, the less successful the model is. A satisfactory correlation between these values was found. Second, on the basis of *k* values calculated with the aid of the estimated parameters, an isorate contour was predicted. This is illustrated in Figure 8 for the inactivation of commercial and self-extracted orange PE. The shape of this isorate contour corresponds well with the shape of the isorate contour based on the experimental *k* values, especially for commercially available orange peel PE. For self-extracted orange PE, deviations were noted in the high-temperature domain. This can be ascribed to the lack of data in the high-temperature region. However, it is experimentally very difficult to obtain data in this region because of the thermal inactivation of orange PE during pressure buildup. Figure 8 also indicates that orange PE is less pressure stable in an acid medium than in water. It can be summarized that the proposed model is able to describe the combined pressure–temperature inactivation of commercial and self-extracted orange PE under the conditions specified.

Combined Pressure–Temperature Inactivation of the Stable Fraction of Commercial Orange Peel PE. To study the effect of pressure and temperature on the inactivation of the stable fraction of commercial orange peel PE, experiments were performed at temperatures ≥70 °C (i.e., 70, 77, 78.5, 80, and 82 °C) combined with pressures ranging from 100 to 900 MPa. Results for pressure experiments at 70 and 80 °C are visualized in Figures 9 and 10, respectively. For treatment at atmospheric pressure, a fast inactivation of the

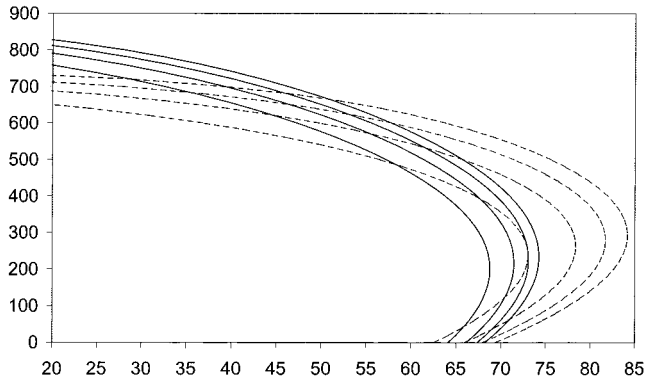

Figure 8. Predicted pressure−temperature kinetic diagram for the *P*/*T* inactivation of commercial orange peel PE in deionized water (full line) and self-extracted orange PE in buffer pH 3.7 (dashed line), based on parameters estimated by eq 12. The inner and outer lines represent *P*/*T* combinations for which $k = 0.02$ and 0.08 min^{-1}, respectively.

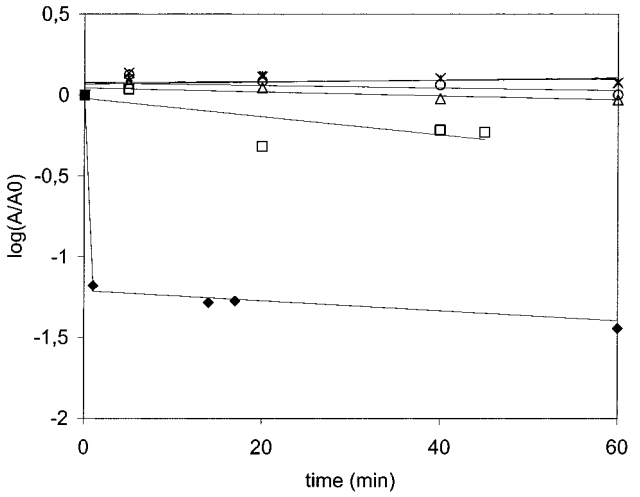

Figure 9. Inactivation of commercial orange peel PE at 70 °C combined with atmospheric pressure (◆), 100 MPa (Δ), 200 MPa (∗), 400 MPa (×), 600 MPa (○), and 900 MPa (□).

heat labile fraction and a slow inactivation of the stable fraction can be derived from these figures. For treatment at elevated pressure, only the decrease of activity of the heat stable fraction can be followed, as during pressure buildup the thermolabile fraction inactivates completely.

For all temperatures studied, an antagonistic effect of pressure and temperature on the inactivation of the heat stable fraction was noticed; that is, the inactivation of the stable fraction was suppressed by increasing pressure. For the lowest temperature studied, that is, 70 °C, an antagonistic effect was observed for pressures up to 600 MPa (see Figure 9). A further increase in pressure accelerated thermal inactivation, and at 900 MPa the inactivation was higher as compared to that at atmospheric pressure. For the other temperatures studied, an antagonistic effect was observed for pressures up to 900 MPa. The principle of microscopic ordering states that at constant temperature, an increase in pressure increases the degree of ordering of the molecules of a substance. On the basis of this principle pressure and temperature are expected to exert an antagonistic effect in molecular terms. However, several deviations from the microscopic principle have been observed for proteins and are discussed by Heremans (1992).

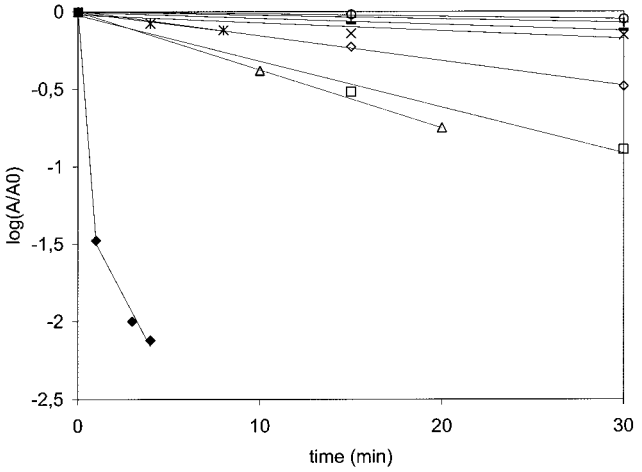

Figure 10. Inactivation of commercial orange peel PE at 80 °C combined with atmospheric pressure (◆), 100 MPa (Δ), 200 MPa (∗), 300 MPa (+), 400 MPa (×), 500 MPa (−), 700 MPa (○), 800 MPa (◇), and 900 MPa (□).

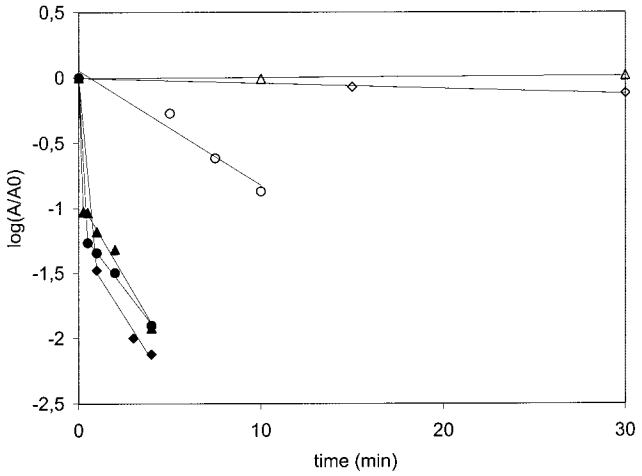

Figure 11. Combined pressure−temperature inactivation of commercial orange peel PE in water at 80 °C (◇), in buffer pH 3.7 at 73 °C (○), and in the presence of Ca^{2+} ions at 82 °C (Δ). Solid symbols refer to atmospheric pressure, and open symbols refer to 500 MPa.

Also, for tomato PE, a clearly antagonistic effect of pressure and temperature was observed at temperatures at which at atmospheric pressure inactivation occurs (Van den Broeck et al., 1999).

Also, the influence of additives on the combined pressure−temperature inactivation of the stable fraction of commercial orange peel PE was evaluated. Similar conditions as for thermal treatment were applied. The influence of Ca^{2+} ions was investigated by dissolving PE (1 mg/mL) in a 1 M $CaCl_2$ solution. To study the influence of an acid medium, commercial orange peel PE (1 mg/mL) was inactivated in a citric acid buffer, 5 mM, pH 3.7. For both additives, an antagonistic effect of pressure and temperature was observed. The antagonistic effect was more pronounced in the presence of a 1 M $CaCl_2$ solution as compared to the inactivation in water, whereas it was less pronounced for the inactivation in acid medium. This is illustrated in Figure 11. In this figure, the inactivation of the stable fraction of commercial orange peel PE in the absence and presence of Ca^{2+} ions, and in an acid medium, is shown for a temperature of, respectively, 80, 82, and 73 °C combined with a pressure of 500 MPa. For these temperatures, a similar inactivation rate constant at atmospheric pres-

sure was estimated for the different environments studied. In this way, the effect of pressure could be compared, starting from the same inactivation rate constant at atmospheric pressure.

CONCLUSION

Pressure and/or temperature inactivation of orange PE was investigated. For both treatments, labile and stable fractions were noted. The stable fraction was ~5% of the initial activity, whether orange PE inactivated by pressure or by temperature.

The stable fraction inactivated at temperatures exceeding 75 °C. Inactivation in acid medium (pH 3.7) accelerated thermal inactivation of the stable fraction as compared to the inactivation in water (pH 4.5), whereas addition of Ca^{2+} ions (1 M) reduced the inactivation rate. At elevated pressure a clearly antagonistic effect of pressure and temperature on the inactivation of the stable fraction was noted, for example, at 80 °C and 900 MPa the inactivation was slower as compared to that at atmospheric pressure. This antagonistic effect was more pronounced in the presence of Ca^{2+} ions (1 M) but less pronounced for the inactivation in an acid medium (pH 3.7).

The labile fraction inactivated in the temperature domain 60−70 °C. Lower temperatures could be applied (15−65 °C) by increasing pressure (up to 900 MPa). On the basis of the estimated kinetic data in the pressure−temperature domain specified, a model predicting the impact of a combined high pressure−temperature process on the inactivation of the labile fraction of orange PE was formulated. This will be helpful in implementing the high-pressure technology in the food industry. However, one should keep in mind the remaining active fraction after pressure−temperature treatment.

NOTATION

a	parameter
b	parameter (MPa^{1-})
c	parameter (MPa^{2-})
d	parameter (MPa^{3-})
e	parameter (MPa^{1-})
f	parameter
A	enzyme activity at time t (mL/min)
A_0	initial enzyme activity (mL/min)
A_∞	enzyme activity when the reaction time is very long (mL/min)
E_a	activation energy (kJ/mol)
k	first-order inactivation rate constant (min^{-1})
k_{atm}	first-order inactivation rate constant at atmospheric pressure (min^{-1})
k_{ref}	first-order inactivation rate constant at a reference temperature (min^{-1})
P	pressure (MPa)
P_{ref}	reference pressure (MPa)
R_p	universal gas constant (=8.314 cm^3·MPa/K·mol)
R_t	universal gas constant (=8.314 J/K·mol)
t	time (min)
T	temperature (K)
T_{ref}	reference temperature (K)
$\Delta V^{\ddagger}$	activation volume (cm^3/mol)

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