Thermal and Combined Pressure−Temperature Inactivation of Orange Pectinesterase: Influence of pH and Additives

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Inactivation of commercially available orange pectinesterase (PE) was investigated under isothermal and isothermal−isobaric conditions. In both cases, inactivation data could be accurately described by a fractional conversion model. The influence of enzyme concentration, pH, Ca^{2+} concentration, and sucrose on the inactivation kinetics was studied. Enzyme stability against heat and pressure increased by increasing enzyme concentration. An increased Ca^{2+} concentration caused sensitization to temperature and increased the residual fraction active PE after thermal treatment. To the contrary, in the case of pressure treatment, decreasing Ca^{2+} concentrations increased pressure inactivation. The remaining fraction active PE after pressure treatment was not influenced by the addition of Ca^{2+} ions. Acidification accelerated thermal as well as pressure−temperature inactivation, whereas in the presence of sucrose an increased temperature and pressure stability of orange PE was observed. Sucrose had no influence on the remaining activity after thermal treatment, but it increased the residual fraction after pressure treatment. The remaining fraction was for all additives studied independent of the pressure and temperature level applied except for the inactivation in an acid medium, when a decrease of the residual fraction was observed with increasing temperature and pressure.

Keywords: *Orange pectinesterase; thermal stability; pressure stability; kinetics; additives*

INTRODUCTION

Pectinesterase (PE) occurs naturally in fruits and vegetables. It is bound by electrostatic interaction to the cell walls. Therefore, on juice extraction, it is technologically impossible to avoid contamination of the juice with this enzyme (Rombouts et al., 1982). In the cloudy juice PE hydrolyzes the pectin (methyl esters of polygalacturonic acid), transforming it gradually into low-methoxy pectin or pectic acids. This de-esterification reduces the juice viscosity, and the low-methoxy pectin that is formed complexes with calcium to form insoluble calcium flocculation, resulting in cloud loss (Krop, 1974).

Pasteurization is the conventional method used to inactivate PE. However, citrus pectinesterase is relatively stable, requiring a severe heat process to inactivate it (Eagerman and Rouse, 1976), which can cause flavor loss and off-flavor formation (Lund, 1977).

In recent years, there has been a growing consumer demand for high-quality juices and fruit-based products. This has led to the investigation of alternative, less severe methods to extend the shelf life of juice products without the flavor and nutrient degradation that occur during thermal pasteurization. One of the most innovative technologies for processing of thermosensitive products is pressure treatment (Farr, 1990; Hayashi, 1989; Mozhaev et al., 1994). As its effect on food ingredients can be related to the fact that mainly noncovalent bonds are affected, food quality factors, such as nutrients, pigments, and flavor, remain mostly unchanged, whereas microorganisms and enzymes are inactivated (Hoover et al., 1989; Knorr, 1993). However, very high pressures are needed to inactivate bacterial spores (Sale et al., 1970) and several enzymes (Seyderhelm et al., 1996; Quaglia et al., 1996; Weemaes et al., 1997, 1998). Therefore, combination with other treatments, such as mild heating, is recommended (Earnshaw, 1995; Farr, 1990).

Research has already been performed on the preservation of citrus fruit juices and the inactivation of orange PE by pressure (Ogawa et al., 1990; Takahashi et al., 1993). Although some promising results have been obtained, more information, mainly kinetic information, is indispensable to optimize the application of this new technology. Therefore, the main objective of the present study was to investigate the high-pressure and/or temperature inactivation kinetics of orange PE. As several papers have already pointed out that PE is only partly inactivated by temperature and pressure, the influence of sensitizing factors which are relevant within the scope of citrus juices such as pH, Ca^{2+} ions, and sucrose was evaluated.

MATERIALS AND METHODS

Pectinesterase and Media. Pectinesterase purified from orange peel [EC 3.1.1.11, Sigma (Bornem, Belgium), product P-5400] (PE) was purchased as a lyophilized powder containing 120 (lot W), 130 (lot X), 160 (lot Z), 205 (lot Y), and 270 (lot V) units per milligram 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

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in deionized water was used as a model system. The pH of this model system was 4.5. To study the effects of enzyme concentration, different concentrations of PE were used. Next to this, the influence of Ca^{2+} concentration was investigated by dissolving PE in 0.5, 1, and 1.5 M $CaCl_2$ solutions. The influence of pH on the inactivation of orange PE was investigated in a citric acid buffer (5 mM). 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. A pH of 3.7 was focused on, which is relevant for orange products. The influence of sucrose was investigated in a 20% sucrose solution.

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

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. 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 (Metrohm). 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). After preset time intervals, the capillaries were withdrawn from the water bath and immediately cooled in ice water. The activity of PE was measured after 10−120 min of storage in ice water. During storage, no reactivation of the enzyme was observed. The temperature range studied varied from 45 to 85 °C.

Isobaric−Isothermal Treatment. The kinetic parameter values for the pressure−temperature inactivation of orange PE were determined on the basis of isobaric−isothermal inactivation experiments and determination of the residual enzyme activity. Hereto, a laboratory pilot scale, multivessel high-pressure equipment (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). The temperature is controlled by a thermostated mantle, which surrounds each vessel and which is connected to a cryostat. 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 micro tubes [0.3 mL, Elkay (Overijse, Belgium)], filled with solution to be treated were enclosed in the pressure vessels, which were equilibrated at a preset temperature. Pressure was built up slowly to minimize adiabatic heating. After reaching the desired pressure, 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 after 10−120 min of storage in ice water. During storage no reactivation of the enzyme was observed. Samples were subjected to pressure treatment at pressures ranging from 400 to 900 MPa, whereas temperatures from 18 to 30 °C were applied.

Data Analysis. Inactivation of enzymes can often be described by a first-order kinetic model

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

where A_0 and A_t are the initial activity and the activity remaining at time t, respectively.

This relation is valid under isothermal (=constant temperature) and isothermal−isobaric (=constant temperature and constant pressure) conditions, whereby the inactivation rate constant k can be determined from a plot of $\ln(A_t/A_0)$ versus time.

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

$$f = (A_0 - A_t)/(A_0 - A_\infty) \tag{2}$$

For most irreversible first-order reactions, A_∞ approaches zero and eq 2 can be reduced to the following:

$$f = (A_0 - A_t)/A_0 \tag{3}$$

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).

$$\ln(A_t/A_0) = \ln(1 - f) = -kt \tag{4}$$

So, it is clear that eq 4 is identical to eq 1 when A_∞ approaches zero.

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

$$\ln(1 - f) = \ln[(A_t - A_\infty)/(A_0 - A_\infty)] = -kt \tag{5}$$

Rearranging eq 5 gives eq 6. By plotting A_t (activity after different time intervals) versus time, inactivation rate constants (k) and remaining activities (A_∞) can be estimated using nonlinear regression on the inactivation data obtained at different temperatures and/or pressures.

$$A_t = A_\infty + (A_0 - A_\infty)\exp(-kt) \tag{6}$$

It should be stressed that for the experiments at a constant temperature and/or pressure, the heating and/or pressurizing time should be long enough so that the remaining activity is no longer changed with respect to time. This nonzero activity may or may not be a function of applied pressure and/or temperature.

Once the inactivation rate constants (k) are known, the temperature and pressure dependence of the rate constant can be estimated. The temperature dependence of k is given by the activation energy (E_a), as indicated in the Arrhenius relationship. Equation 7 gives the linearized form of this relationship.

$$\ln k = \ln k_{ref} + \left[\frac{E_a}{R_t}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{7}$$

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 atmospheric pressure, and we assume that this relation is also valid at elevated pressure.

The pressure dependence of k is expressed by the activation volume (V_a), as presented in eq 8 in linearized form (Morild, 1981), and can be obtained through linear regression when

Table 1. Kinetic Parameters for Isothermal Inactivation of Orange PE in Water at Atmospheric Pressure

	lot V		lot X	lot Y	lot Z	
	0.4 mg of solid/mL	1.0 mg of solid/mL	0.5 mg of solid/mL	0.8 mg of solid/mL	1.0 mg of solid/mL	1.5 mg of solid/mL
inactivation rate constant (min^{-1})						
57 °C	0.059 ± 0.005^a	0.041 ± 0.005^a				
60 °C	0.153 ± 0.005^a	0.103 ± 0.003^a	0.124 ± 0.003^a	0.112 ± 0.008^a	0.165 ± 0.003^a	0.113 ± 0.004^a
63 °C	0.507 ± 0.026^a	0.266 ± 0.013^a	0.317 ± 0.006^a	0.397 ± 0.028^a	0.469 ± 0.012^a	0.335 ± 0.014^a
65 °C	0.942 ± 0.026^a	0.522 ± 0.029^a	0.748 ± 0.027^a	0.680 ± 0.042^a	0.897 ± 0.084^a	0.591 ± 0.033^a
67 °C			1.415 ± 0.024^a	1.269 ± 0.031^a	2.359 ± 0.126*	1.396 ± 0.055^a
% residual activity	5.9 ± 1.0^a	6.0 ± 1.3^a	5.4 ± 0.3^a	6.9 ± 0.9^a	16.3 ± 0.6^a	19.2 ± 0.6^a
E_a (kJ/mol)	326.9 ± 11.7^b	301.4 ± 12.3^b	332.9 ± 15.0^b	323.6 ± 22.3^b	350.5 ± 22.8^b	331.9 ± 16.1^b

[a] Asymptotic standard error. [b] Standard error.

the logarithm of the rate constant is plotted versus pressure. This relation is valid at constant temperature.

$$\ln k = \ln k_{atm} - (V_a P/R_p T) \quad (8)$$

RESULTS AND DISCUSSION

Thermal Inactivation Kinetics. *Effect of Enzyme Concentration.* Isothermal inactivation of orange PE in deionized water was studied in the temperature range from 57 to 67 °C. Different concentrations of different lots were taken into consideration. In this temperature domain, only the heat labile fraction inactivated, whereas the heat stable fraction remained active. Therefore, the fractional conversion model could be used to estimate the kinetic inactivation parameters, that is, the inactivation rate constant k of the heat labile fraction and the remaining percentage of active enzyme. These parameters are presented in Table 1.

The influence of initial concentration on the inactivation rate constant k of the labile fraction seems to be dependent on the lot. This implies that the same lot should be considered to draw conclusions about the effect of enzyme concentration. By comparing 0.4 and 1.0 mg of solid/mL of lot V and 1.0 and 1.5 mg of solid/mL of lot Z, it can be concluded that the enzyme stability against heat increases by increasing the enzyme concentration. The temperature dependence of the inactivation rate constant k, expressed by the activation energy E_a, slightly decreases with increasing enzyme concentration. However, this effect is not pronounced. For lot Z, an increase in the residual fraction was observed with increasing enzyme concentration. A similar increase was not found for lot V.

Although differences were observed among the different lots, a common characteristic of all the lots used in this study was a remaining fraction being independent of the temperature level in the temperature domain studied; that is, the stable fraction was not inactivated for temperatures up to 67 °C. This is visualized in Figure 1 for the thermal inactivation of orange PE (lot Z) in a concentration of 1.5 mg of solid/mL. The heat stable fraction could be inactivated completely by increasing the temperature to 85 °C during 1 min.

The percentage residual PE is dependent on the lot used. Lot Z is clearly characterized by a higher heat stable fraction than the other lots. Sixteen to 19% of the original activity, depending on the initial enzyme concentration of lot Z, remained active after treatment, whereas for lots X, Y, and V, 5−7% of the original activity was found to be thermostable. It was assumed that the residual fraction active PE was a reflection of the heterogeneity of the enzyme. Several studies mention the different heat stability of different fractions of orange PE (Versteeg et al., 1980; Rothschild et al., 1975;

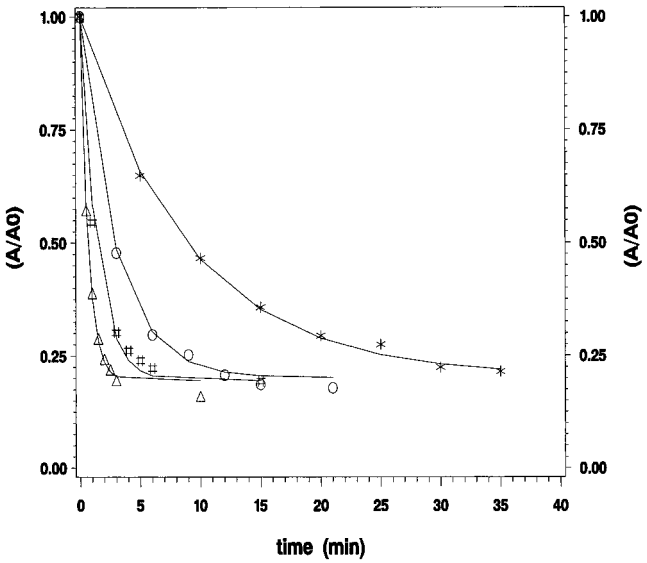

Figure 1. Thermal inactivation of orange PE (1.5 mg of solid/mL of lot Z) in water at 60 (*), 63 (○), 65 (#), and 67 °C (Δ).

Wicker and Temelli, 1988; Snir et al., 1996). In these studies a break in the inactivation curve at a certain temperature was observed where residual activity was not destroyed until higher temperatures were applied.

Quantification of relative ratios of thermostable PE to total PE remains elusive. Versteeg et al. (1980) reported that the most thermostable form in navel oranges accounted for only 5% of total PE activity. Rombouts et al. (1982) found a total of 12 isozymes in different citrus cultivars. According to them, thermostable PE represented 6% of total activity in navel oranges, 11% in Salustiana oranges, 7% in Shamouti oranges, 9% in lemons, 33% in grapefruits, and 10% in mandarins. Also Van den Broeck et al. (1999) reported that the percentage of residual PE is dependent on the variety of the oranges used to extract PE. Snir et al. found that the percentage of thermostable PE varied greatly (from 0 to 18.6%) between and within samples of eight types of citrus cultivars (Snir et al., 1996). Some differences may be related to experimental differences in protocol (Snir et al., 1996; Wicker et al., 1988), geographic location, growth practice, postharvest handling, seasonal differences, and other parameters (Snir et al., 1996). They could also be due to factors that affect solubilization of PE from an inactive pectin complex (Snir et al., 1995).

Influence of pH. The influence of pH on the isothermal inactivation of orange PE was investigated by using a 5 mM citric acid buffer. As pH, the pH of commercially available orange juice was selected, that is, pH 3.7. The inactivation kinetics of two different lots (1 mg of solid/

Table 2. Kinetic Parameters for Isothermal Inactivation of Orange PE in Water and Citric Acid Buffer (pH 3.7) at Atmospheric Pressure

	lot Z (1 mg of solid/mL)		lot W (1.7 mg of solid/mL)
	water, pH 4.5	buffer, pH 3.7	buffer, pH 3.7
inactivation rate constant (min^{-1}), % residual activity			
45 °C			0.184 ± 0.006^a, 7.4 ± 0.6^a
50 °C		0.579 ± 0.073^a, 21.2 ± 1.7^a	0.951 ± 0.090^a, 5.6 ± 1.2^a
55 °C		1.158 ± 0.141^a, 13.2 ± 1.0^a	4.530 ± 0.453^a, 5.2 ± 1.0^a
57 °C			6.376 ± 1.184^a, 4.4 ± 1.2^a
60 °C	0.165 ± 0.003^a, 16.3 ± 0.6^a	2.375 ± 0.267^a, 12.2 ± 1.0^a	
63 °C	0.469 ± 0.012^a, 16.3 ± 0.6^a		
65 °C	0.897" 0.084^a, 16.3 ± 0.6^a		
67 °C	2.359 ± 0.126^a, 16.3 ± 0.6^a		
E_a (kJ/mol)	350.5 ± 22.8^b	126.2 ± 2.4^b	263.0 ± 12.3^b

[a] Asymptotic standard error. [b] Standard error.

mL of lot Z and 1.7 mg of solid/mL of lot W) were considered. Temperatures ranged from 45 to 60 °C. In this temperature range, only the heat labile fraction inactivated. The inactivation rate constant of the labile fraction and the remaining percentage active PE were estimated by fractional conversion kinetics. The estimated values for the inactivation of orange PE in water and in acid buffer are presented in Table 2. By analogy with the inactivation in water is the inactivation behavior in citric acid buffer clearly dependent on the lot used. Again, lot Z is characterized by a higher percentage of residual PE after treatment.

A comparison of the kinetic parameters obtained for lot Z, reveals that orange PE inactivates more rapidly and at lower temperatures in citric acid buffer (pH 3.7) than in water (pH 4.5), which means that orange PE is less thermostable in an acid medium. 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. The increased inactivation of orange PE with lowering pH is confirmed by literature data. Rothschild et al. (1975) reported that to inactivate PE in grapefruit juice, the temperature could be lowered from 90 to 80 °C with decreasing pH from 3.4 to 2.8.

It can also be concluded that the inactivation rate constant in citric acid buffer (pH 3.7) is significantly less temperature sensitive than the inactivation rate constant in water. The temperature dependence of the inactivation rate constant k is visualized in Figure 2.

Regarding the residual fraction active PE, this fraction is obviously more temperature dependent in buffer than in water. A decrease of the residual fraction is observed with increasing temperature; 21, 13, and 12% of the original activity of lot Z remained active after treatment at, respectively, 50, 55, and 60 °C. This indicates that the residual fraction active PE after treatment in a similar temperature domain is smaller in buffer than in water.

Orange PE from lot W is even more thermolabile, and a smaller fraction remains active after treatment.

Influence of Ca^{2+} Ions. To investigate the influence of Ca^{2+} ions on the thermal inactivation of orange PE, the enzyme was dissolved in 0.5, 1, and 1.5 M $CaCl_2$ solution. Temperatures from 45 to 58 °C were applied. In all cases, inactivation data could be modeled with the fractional conversion model. The estimated kinetic parameters for the inactivation of orange PE in the absence and presence of Ca^{2+} ions are presented in Table 3. In Figure 3, the inactivation of orange PE (1.5 mg of solid/mL of lot Z) in 1.0 M $CaCl_2$ solution is shown. This figure clearly illustrates that the residual fraction

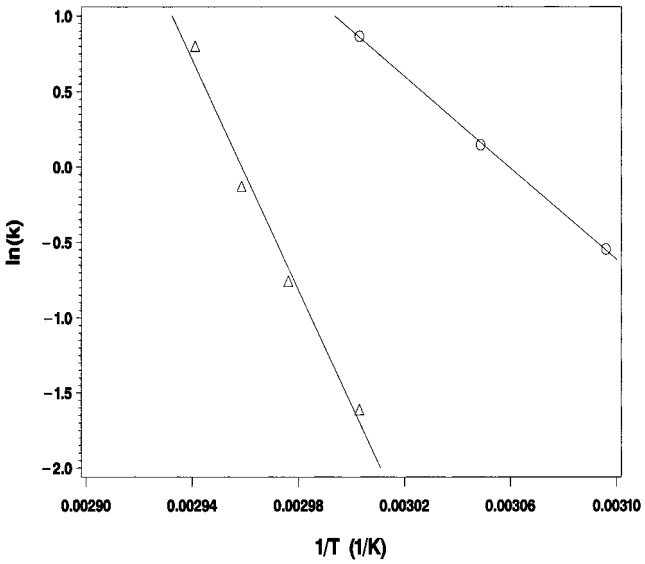

Figure 2. Temperature dependence of the inactivation rate constants for thermal inactivation of orange PE (1 mg of solid/mL of lot Z) in citric acid buffer (pH 3.7) (○) and in water (Δ).

is independent of the temperature level in the temperature domain studied.

By comparing the inactivation parameters of lot Z (see Table 3), it can be deduced that Ca^{2+} ions decrease the thermal stability of PE. Moreover, in the case of 1.0 and 1.5 M $CaCl_2$ solution, a decreased temperature sensitivity of the inactivation rate constant k was noticed. Although orange PE is less thermostable in the presence of Ca^{2+} ions, the percentage of residual PE after treatment is higher. Comparing the different molarities of $CaCl_2$ solution mutually shows that increasing the Ca^{2+} concentration decreases the thermal stability of orange PE, lowers the inactivation temperature, and results in a larger percentage residual PE after treatment (respectively, 23, 28, and 36% for 0.5, 1, and 1.5 M $CaCl_2$ solution). Also, a small decreased temperature sensitivity of the inactivation rate constant k is observed with increasing salt concentration. To be sure that the significant increase in residual fraction active PE after treatment is not dependent on the lot used, similar experiments were performed with lot V (0.4 mg of solid/mL). Again an increased inactivation, a decreased activation energy, and an increased residual fraction active PE after treatment were observed in a 1 M $CaCl_2$ solution. However, it should be stressed that the increase in residual fraction is less pronounced for lot V.

It is also worthwhile mentioning that the initial activity of orange PE in the presence of Ca^{2+} ions

Table 3. Kinetic Parameters for Isothermal Inactivation of Orange PE in the Presence and Absence of $CaCl_2$ at Atmospheric Pressure

	lot Z (1.5 mg of solid/mL)				lot V (0.4 mg of solid/mL)	
	absence of $CaCl_2$	0.5 M $CaCl_2$	1.0 M $CaCl_2$	1.5 M $CaCl_2$	absence of $CaCl_2$	1.0 M $CaCl_2$
inactivation rate constant (min^{-1})						
45 °C				0.142 ± 0.004^a		0.114 ± 0.008^a
47 °C				0.271 ± 0.009^a		
50 °C			0.259 ± 0.003^a	0.861 ± 0.069^a		0.716 ± 0.055^a
52 °C						1.260 ± 0.136^a
53 °C		0.224 ± 0.002^a	0.789 ± 0.022^a			
55 °C		0.486 ± 0.009^a	1.515 ± 0.046^a	4.315 ± 0.293^a		
58 °C		1.429 ± 0.013^a				
60 °C	0.113 ± 0.004^a				0.153 ± 0.005^a	
63 °C	0.335 ± 0.014^a				0.507 ± 0.026^a	
65 °C	0.591 ± 0.033^a				0.942 ± 0.026^a	
67 °C	1.396 ± 0.055^a					
% residual activity	19.2 ± 0.6^a	23.3 ± 0.1^a	28.0 ± 0.2^a	36.4 ± 0.5^a	5.9 ± 1.0^a	9.8 ± 1.7^a
E_a (kJ/mol)	331.9 ± 16.1^b	331.9 ± 5.4^b	312.6 ± 9.9^b	298.5 ± 6.9^b	326.9 ± 11.7^b	267.6 ± 5.9^b

[a] Asymptotic standard error. [b] Standard error.

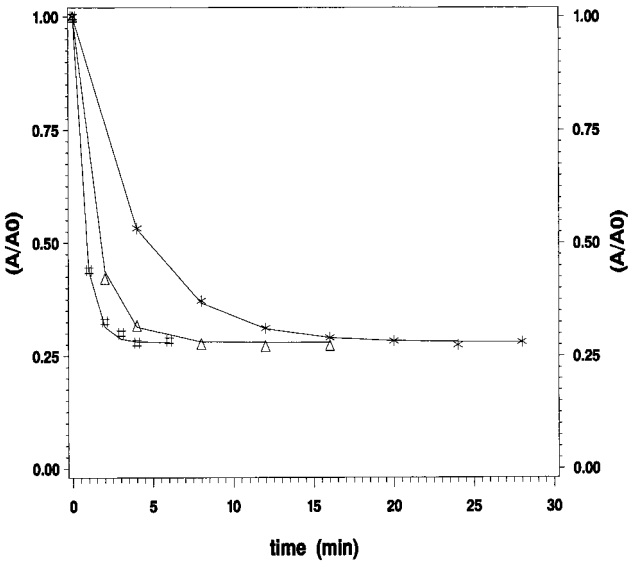

Figure 3. Thermal inactivation of orange PE (1.5 mg of solid/mL of lot Z) in the presence of $CaCl_2$ (1 M) at 50 (*), 53 (Δ), and 55 °C (#).

decreases as the molarity of the $CaCl_2$ solution increases. This can be explained by a competitive displacement at higher calcium concentrations for PE binding sites on pectin (Snir et al., 1995). Carboxylate groups located in the vicinity of the $-COO-CH_3$ groups that are subjected to hydrolysis by PE are required to allow the hydrolysis to occur. These carboxylate groups interact with the active site of the enzyme (Rexova-Benkova and Markovic, 1976). Binding of metal ions to these groups therefore inhibits the enzyme activity, at high salt concentration (Nari et al., 1991). The competition of orange PE binding sites on pectin by calcium is also illustrated by Charnay et al. (1992) using Lineweaver–Burk plots. Low concentrations of ions, on the other hand, can activate the enzyme. This effect is also ascribed to an interaction of the ions with the substrate rather than with the enzyme. Blocks of carboxylate groups may trap enzyme molecules, preventing them from reacting with the $-COO-CH_3$ groups to be hydrolyzed (Nari et al., 1991). Metal ions decrease this inhibition by binding to carboxylate groups of the pectin.

Influence of Sucrose. The influence of sucrose on the thermal inactivation of commercially available orange PE was investigated by heating orange PE (0.4 mg of solid of lot V/mL) in a 20% sucrose solution. Temperatures from 60 to 65 °C were applied. The fractional conversion model was the most suitable model to fit the inactivation data. The estimated kinetic parameters in the presence of and without sucrose are presented in Table 4.

Table 4. Kinetic Parameters for Isothermal Inactivation of Orange PE in the Presence and Absence of 20% Sucrose at Atmospheric Pressure

	lot V (0.4 mg solid/mL)	
	absence of 20% sucrose	presence of 20% sucrose
inactivation rate constant (min^{-1})		
60 °C	0.153 ± 0.005^a	0.072 ± 0.007^a
61.5 °C		0.121 ± 0.009^a
63 °C	0.507 ± 0.026^a	0.239 ± 0.016^a
65 °C	0.942 ± 0.026^a	0.440 ± 0.015^a
% residual activity	5.9 ± 1.0^a	5.9 ± 1.0^a
E_a (kJ/mol)	326.9 ± 11.7^b	346.3 ± 18.4^b

[a] Asymptotic standard error. [b] Standard error.

From a comparison of the inactivation parameters of lot V (see Table 4), it appears that the inactivation rate decreases in the presence of sucrose. However, the decreased stability does not influence the residual fraction: the same fraction remained active as for the inactivation in absence of sucrose. Estimates for the E_a values indicate that the inactivation rate constant k is slightly more temperature sensitive in the presence of sucrose.

Pressure–Temperature Inactivation Kinetics. *Effect of Enzyme Concentration.* Different concentrations of different lots of orange PE were subjected to combined pressure–temperature treatment. The pressure range studied varied from 600 to 900 MPa, whereas temperatures of 20, 25, and 30 °C were applied. Also for combined pressure–temperature treatment, a certain fraction of orange PE appears to be stable, and hence the fractional conversion model was used to fit the inactivation data. The estimated kinetic parameters are presented in Table 5.

From this table, the synergistic effect of pressure and temperature on the inactivation of the labile fraction can be derived; increasing pressure at a fixed temperature increases the inactivation rate (Table 5). Likewise, increasing temperature at a fixed pressure leads to increased inactivation rates. In Table 5, the residual fraction is expressed as a percentage of the original activity. Once more, a distinction can be made between lot Z and the other lots with regard to the remaining

Table 5. Kinetic Parameters for Isothermal–Isobaric Inactivation of Orange PE in Water at Elevated Pressure

	lot V		lot Y	lot Z		lot X	
	0.4 mg of solid/mL, 25 °C	1.0 mg of solid/mL, 25 °C	0.8 mg of solid/mL, 30 °C	1.0 mg of solid/mL, 30 °C	1.5 mg of solid/mL, 30 °C	1.8 mg of solid/mL, 20 °C	1.8 mg of solid/mL, 30 °C
inactivation rate constant (min^{-1})							
600 MPA			0.029 ± 0.003^a	0.020 ± 0.002^a	0.024 ± 0.001^a		0.018 ± 0.001^a
700 MPA			0.046 ± 0.005^a	0.061 ± 0.003^a			
750 MPA	0.072 ± 0.002^a	0.047 ± 0.003^a					
800 MPA	0.119 ± 0.003^a	0.073 ± 0.006^a	0.139 ± 0.024^a			0.130 ± 0.011^a	0.196 ± 0.007^a
850 MPA	0.241 ± 0.016^a	0.117 ± 0.004^a					
900 MPA	0.295 ± 0.014^a			0.586 ± 0.243^a			
% residual activity	4.9 ± 0.6^a	5.3 ± 0.7^a	5.3 ± 0.7^a	14.0 ± 1.9^a	27.9 ± 1.4^a	7.0 ± 0.8^a	6.6 ± 0.5^a
V_a (cm^3/mol)	-24.55 ± 3.44^b	-22.48 ± 0.61^b	-27.52 ± 0.60^b	-28.23 ± 0.16^b			

[a] Asymptotic standard error. [b] Standard error.

Table 6. Kinetic Parameters for Isothermal–Isobaric Inactivation of Orange PE in Citric Acid Buffer (pH 3.7) at Elevated Pressure

	lot W (1.7 mg of solid/mL)				lot V (0.4 mg of solid/mL)
	18 °C	23 °C	28 °C	E_a (kJ/mol)	25 °C
inactivation rate constant (min^{-1}), % residual activity					
400 MPa	0.024 ± 0.003^a, 7.0 ± 1.2^a	0.032 ± 0.002^a, 7.0 ± 1.2^a	0.056 ± 0.001^a, 7.0 ± 1.2^a	62.95 ± 11.11^b	
450 MPa					0.027 ± 0.004^a, 30.5 ± 3.7^a
500 MPa	0.107 ± 0.008^a, 6.9 ± 0.5^a	0.138 ± 0.005^a, 6.9 ± 0.5^a	0.208 ± 0.011^a, 6.9 ± 0.5^a	48.42 ± 6.97^b	0.035 ± 0.004^a, 12.8 ± 4.7^a
550 MPa			0.323 ± 0.027^a, 7.8 ± 0.5^a		
600 MPa	0.229 ± 0.015^a, 4.3 ± 0.6^a	0.317 ± 0.026^a, 4.3 ± 0.6^a			0.108 ± 0.009^a, 3.2 ± 1.9^a
V_a (cm^3/mol)	-27.08 ± 5.18^b	-27.76 ± 4.41^b	-29.29 ± 2.65^b		-23.43 ± 3.89^b

[a] Asymptotic standard error. [b] Standard error.

active fraction: lot Z is characterized by a higher pressure stable fraction. However, the remaining fraction was of the same order of magnitude for each lot individually, whether orange PE was inactivated by temperature or by pressure.

Similar to thermal treatment was the influence of initial concentration on the inactivation kinetics dependent on the lot used. By considering a single lot (lot V), it can be derived that the enzyme is more pressure stable in more concentrated solutions. However, in the case of very low inactivation (see lot Z), enzyme concentration had almost no influence on the inactivation rate constant *k*. Moreover, a small decreased pressure sensitivity of the inactivation rate constant *k*, expressed by the activation volume, was observed with increasing enzyme concentration. Regarding the residual fraction active PE after pressure treatment, differences were observed depending on the lot. For lot Z, an increase in residual fraction from 14 to 28% of the original activity was noticed with increasing enzyme concentration. However, for lot V, the residual fraction remained the same (i.e., 5%). A similar behavior was observed for thermal inactivation.

Because no complete inactivation of the enzyme was attained after pressure treatment, it can be concluded that the PE isozymes with high heat resistance may also show pressure resistance. However, to elucidate the relation between the pressure and temperature resistance of this fraction, more experiments are needed.

Also from literature data, it appears that orange PE can only be partially inactivated by pressure treatment. This implies that orange juices, pasteurized by pressure treatment, should be stored under refrigerated conditions to minimize cloud loss. Parish et al. (1994) found 94, 55, 33, 20, and 13% of the original PE activity after treatment of orange juice during 30 s at, respectively, 500, 600, 700, 800, and 900 MPa. They concluded that high-pressure treatment substantially reduced PE activity but that levels of activity in the range found after treatment with the highest pressure used still can destabilize citrus cloud. Ogawa et al. (1992) found 10–20% of the original activity after treatment of orange juice at 23 °C and 600 MPa during 10 min. This is in contrast to a study of Basak and Ramaswamy (1996). According to them, no residual fraction was observed after pressure inactivation of citrus PE. They analyzed their data in terms of a dual effect of pressure: the first one, designated an instantaneous pressure kill (IPK), depended only on the pressure level; a second one, which depended on the holding time at each pressure level, is described by first-order reaction kinetics.

Influence of pH. The influence of pH on the isothermal–isobaric inactivation of orange PE (1.7 mg of solid of lot W/mL, 0.4 mg of solid of lot V/mL) was investigated in a 5 mM citric acid buffer (pH 3.7). The kinetic parameters estimated with the aid of the fractional conversion model are presented in Table 6. Figure 4 illustrates the pressure inactivation of orange PE (0.4 mg of solid of lot V/mL) in citric acid buffer (pH 3.7) at a temperature of 25 °C.

From a comparison of the kinetic parameters of lot W, estimated for the inactivation in an acid medium at atmospheric pressure and elevated pressure (Tables 2 and 6), the synergistic effect of pressure and temperature is clear. An inactivation rate constant similar to that at 45 °C can be obtained at room temperature if pressure is increased to 500 MPa. Increasing pressure at a fixed temperature and increasing temperature at a fixed pressure increase the inactivation rate. A comparison of both tables also reveals that the inactivation rate constant *k* is less temperature sensitive at elevated pressure. A similar decreased temperature sensitivity of the inactivation rate constant *k* at elevated pressure was also noticed for *Bacillus subtilis* α-amylase (Ludikhuyze et al., 1996), trypsin (Miyagawa et al., 1963), and polyphenol oxidase (Weemaes et al., 1997). Moreover, a small decrease in activation energy is observed with increasing pressure. Regarding the activation volume, this parameter is only slightly influenced by temperature. The remaining fraction is dependent on the pressure level, whereas no differences were observed with respect to the temperature level in the

Table 7. Kinetic Parameters for Isothermal−Isobaric Inactivation of Orange PE in the Presence and Absence of $CaCl_2$ at Elevated Pressure and 25 °C

	lot V (0.4 mg of solid/mL)			
	absence of $CaCl_2$	0.5 M $CaCl_2$	1.0 M $CaCl_2$	1.5 M $CaCl_2$
inactivation rate constant (min^{-1})				
750 MPA	0.072 ± 0.002[a]	0.113 ± 0.019[a]	0.089 ± 0.008[a]	0.053 ± 0.004[a]
800 MPA	0.119 ± 0.003[a]	0.148 ± 0.005[a]	0.146 ± 0.010[a]	0.097 ± 0.012[a]
850 MPA	0.241 ± 0.016[a]		0.279 ± 0.014[a]	
860 MPA		0.455 ± 0.037[a]		0.172 ± 0.012[a]
900 MPa	0.295 ± 0.014[a]			
% residual activity	4.9 ± 0.6[a]	5.5 ± 1.3[a]	4.1 ± 0.9[a]	5.0 ± 0.9[a]
V_a (cm^3/mol)	−24.55 ± 3.44[b]	−31.80 ± 9.42[b]	−28.26 ± 2.34[b]	−26.35 ± 1.64[b]

[a] Asymptotic standard error. [b] Standard error.

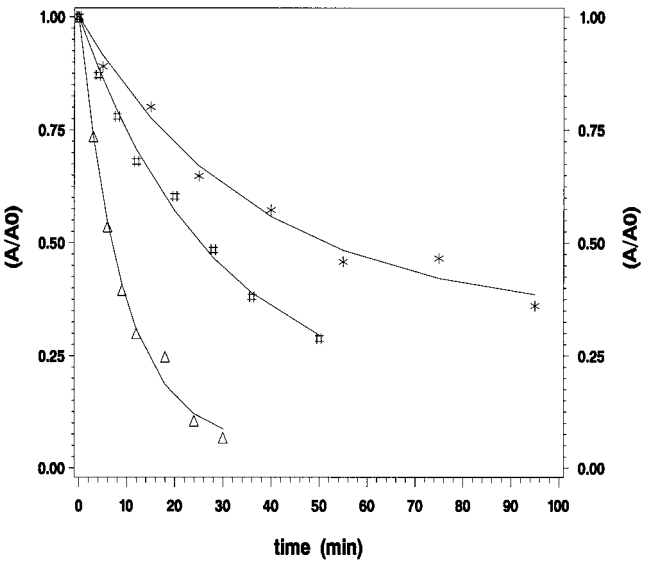

Figure 4. Pressure inactivation of orange PE (0.4 mg of solid/mL of lot V) in citric acid buffer (pH 3.7) at a temperature of 25 °C in combination with pressures of 450 (*), 500 (#), 600 MPa (Δ).

temperature domain studied. The lowest residual fraction was obtained at the highest pressure level.

A comparison of the kinetic parameters of lot V for the inactivation in water and in buffer at elevated pressure (Tables 5 and 6) reveals that the enzyme is less pressure stable in an acid medium. Besides this, also a small decrease of the pressure sensitivity of the inactivation rate constant k was observed. As for thermal inactivation, the residual fraction is dependent on the pressure applied. The remaining fraction decreased from 31 to 3% when the pressure was increased from 450 to 600 MPa. This implies that a smaller residual fraction can be obtained in buffer as compared to water at a lower pressure level.

Influence of Ca^{2+} Ions. The influence of Ca^{2+} ions on the pressure inactivation of orange PE (0.4 mg of solid of lot V/mL) at room temperature was investigated in an 0.5, 1, and 1.5 M $CaCl_2$ solution. The kinetic parameters, estimated with the aid of the fractional conversion model, for the inactivation of orange PE in the presence of and without Ca^{2+} ions, are presented in Table 7.

A comparison of the kinetic data indicates that an increased pressure inactivation was observed for the lower molarities (0.5 and 1.0 M), whereas in the presence of a 1.5 M $CaCl_2$ solution, a decreased inactivation was noticed. This is in contrast to thermal treatment, when an increased inactivation was observed for each of the Ca^{2+} concentrations studied. Also, a slight increase in the pressure sensitivity of the inactivation rate constant k was observed, as compared to the inactivation in absence of Ca^{2+} ions. Notwithstanding the altered stability and sensitivity of orange PE, the remaining active fraction remained nearly the same: 4−5% of the original activity was found to be active after pressure treatment in the absence as well as in the presence of Ca^{2+} ions.

A comparison of the different molarities mutually points out that increasing the salt concentration increases the pressure stability and decreases the pressure sensitivity of the inactivation rate constant k. However, no increase or decrease in residual fraction was observed after pressure treatment with respect to different Ca^{2+} concentrations.

Table 8. Kinetic Parameters for Isothermal−Isobaric Inactivation of Orange PE in the Presence and Absence of 20% Sucrose at Elevated Pressure and 25 °C

	lot V (0.4 mg of solid/mL)	
	absence of 20% sucrose	presence of 20% sucrose
inactivation rate constant (min^{-1})		
750 MPA	0.072 ± 0.002[a]	0.024 ± 0.004[a]
800MPA	0.119 ± 0.003[a]	0.050 ± 0.003[a]
850 MPA	0.241 ± 0.016[a]	0.071 ± 0.005[a]
900 MPA	0.295 ± 0.014[a]	0.130 ± 0.006[a]
% residual activity	4.9 ± 0.6[a]	8.3 ± 1.4[a]
V_a (cm^3/mol)	−24.55 ± 3.44[b]	−26.67 ± 2.40[b]

[a] Asymptotic standard error. [b] Standard error.

Influence of Sucrose. The influence of sucrose on the pressure stability of orange PE (0.4 mg of solid of lot V/mL) at room temperature was investigated in a 20% sucrose solution. The kinetic parameters, estimated with the aid of the fractional conversion model, are presented in Table 8.

A comparison of the data reveals that the pressure inactivation decreased in the presence of sucrose. A similar behavior was found for thermal inactivation. Also, an increased pressure sensitivity of the inactivation rate constant k and an increased residual fraction active PE after pressure treatment were observed in the presence of sucrose.

CONCLUSION

This study reveals that orange PE can only be partly inactivated by temperature as well as by combined pressure−temperature treatment. Therefore, heat and pressure inactivation of orange PE was modeled by fractional conversion kinetics to estimate the inactivation rate constant of the labile fraction and the remaining percentage of active PE. A complete inactivation of orange PE could be attained by thermal treatment (1 min at 85 °C).

It was also noted that inactivation kinetics are clearly dependent on enzyme concentration, pH, sucrose, and Ca^{2+} ions. Enzyme stability against heat and pressure is higher in concentrated than in diluted solutions. Moreover, depending on the lot used, a larger percentage of residual PE is observed after temperature and pressure treatment in more concentrated solutions. With regard to the influence of Ca^{2+} ions, it can be summarized that this additive has a clearly different influence on pressure and temperature stability of orange PE. Increasing the salt concentration increases the pressure stability, whereas it decreases the temperature stability. A higher salt concentration results in a higher residual fraction of active PE after thermal treatment. However, no increase or decrease in residual fraction is observed after pressure treatment with respect to different Ca^{2+} concentrations.

Addition of sucrose increases the pressure and temperature stability of orange PE. It has no influence on the remaining active fraction after thermal treatment, whereas after pressure treatment, an increased remaining fraction is noted. Acid media accelerate thermal as well as pressure inactivation. In this medium is the residual fraction dependent on temperature and pressure. However, for all other additives studied, the remaining fraction is independent of temperature and pressure levels applied.

ABBREVIATIONS USED

A_t, activity at time t (mL/min); A_0, initial activity (mL/min); A_∞, 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); V_a, activation volume (cm^3/mol).

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