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Inactivation Kinetics of Purified Tomato Polygalacturonase by Thermal and High-Pressure Processing

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Tomato polygalacturonase (PG) was extracted from ripe tomatoes and purified by cation exchange and gel filtration chromatography. Cation exchange chromatography yielded two peaks with PG activity: the first peak was identified as PG2 (the heat labile form) and the second one as PG1 (the heat stable form). Both PG2 and PG1 presented a molar mass of 42 kDa when analyzed by SDS-PAGE and an isoelectric point >9.3. Thermal inactivation of purified tomato PG2, at pH 4.4, in the temperature range from 53 to 63 °C, followed first-order kinetics. Combined pressure−temperature inactivation of tomato PG2 was studied at 5−55 °C/100−600MPa. Under all pressure−temperature conditions, PG2 inactivation followed first-order kinetics. Purified tomato PG1, although more thermostable than PG2, showed a pressure stability very similar to that of PG2. These results indicate that high-pressure processing is an efficient alternative to inactivate tomato PG without the need for applying high temperatures.

KEYWORDS: Tomato; polygalacturonase; thermal; high pressure; kinetics

INTRODUCTION

Polygalacturonase (PG) (EC 3.2.1.15), a cell wall bound enzyme, is present in many fruits and vegetables, for example, tomato fruit (*1*). Through different purification steps of tomato extract, two fractions with PG activity can be obtained: the heat labile form PG2 (totally inactivated at 65 °C for 5 min) and the heat stable form PG1 (stable at 65 °C for 5 min) (*2*−*4*). Ali and Brady (*5*) separated the heat labile form PG2 by gel electrophoresis into PG2A and PG2B. Both PG1 and PG2 present the same polypeptide chain; PG1 consists of PG2 interacting with a heat stable protein (β-subunit), which transforms the heat labile PG2 into the heat stable form PG1 (*4*, *6*, *7*). It is still under discussion whether PG1 exists in vivo or is to be considered as an artifact formed during extraction (*4*, *8*, *9*). In ripe tomato fruits the most abundant form is PG2 (*5*, *10*, *11*). PG catalyzes the hydrolytic cleavage of the α-(1→4)-glycosidic bonds in the D-galacturonan moiety of pectic substances. This reaction results in lower molecular weight chains of galacturonic acid, which will induce a marked decrease of viscosity of tomato-based products (*12*). For this reason PG is normally inactivated by thermal processing (*13*). Nonthermal technologies such as high-pressure processing have been introduced or are currently intensively studied because they offer the advantages of improved food quality retention including color, flavor, and nutritional values while inactivating enzymes and vegetative cells (*14*, *15*). Until now, little attention has been paid to the study of PG inactivation kinetics by either thermal or pressure processing (*16*−*18*). Studies on purified systems as a basis to explain in situ studies are completely absent. In this study, detailed thermal and high-pressure inactivation kinetics of purified PG2 at pH 4.4 are described.

MATERIALS AND METHODS

Raw Material Preparation. A batch of tomatoes (*Lycopersicon esculentum* L. cv. Flandria Prince) was purchased at commercial maturity. The tomatoes were washed, cut in small pieces, frozen in liquid nitrogen, and stored in a freezer at −80 °C. Before use, they were thawed at room temperature. Thawed tomatoes (500 g) were blended with cold distilled water (1:1 w/v) for 1 min, the pH was adjusted to 3.0 with 0.1 M HCl, and the tomatoes were stirred for 15 min. The solution was centrifuged at 8000*g* for 20 min. The same procedure was repeated using the pellets but with the addtion of 1:1.5 (w/v) distilled water.

Extraction Procedure. PG was extracted using a modified method of Pressey (*19*). PG extraction of the final pellet was performed with 1.2 M NaCl (1:1 w/v), the pH was adjusted to 6.0 with 0.1 M NaOH, and the mixture was stirred for 3 h. The solution was centrifuged at 10000*g* for 20 min and the supernatant subjected to ammonium sulfate precipitation. The fraction precipitating between 30 and 80% saturation was dissolved in 40 mL of 0.5 M NaCl and dialyzed against distilled water for 3 h. The extracted solution was kept at −25 °C for further use. All steps were performed at 4 °C.

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Purification of Tomato Polygalacturonase. The purification procedure was based on the method described by Pogson et al. (*20*). The purification was performed with a low-pressure chromatography system (Äkta Prime, Amersham Biosciences, Uppsala, Sweden), and parameters such as pH, conductivity, temperature, pressure, and UV absorption (at 280 nm) were constantly monitored during separations. Crude tomato PG extract (~5 mL) was thawed at room temperature, filtered using a 0.45 μm syringe-driven filter (Millipore, Bedford, MA), and applied on a cation exchange column (CIEX, Hi Prep 16/10 SP/XL, Amersham Biosciences). The column was previously equilibrated with 50 mM sodium acetate buffer, pH 4.4, and the elution was performed with a linear salt gradient to 1.0 M NaCl in sodium acetate buffer, pH 4.4, at a flow rate of 0.5 mL/min for 10 column volumes. The separation was carried out at ambient temperature. Fractions of 4 mL were collected and assayed for polygalacturonase (PG) and pectinmethylesterase (PME) activity. Fractions showing PG activity were pooled and concentrated (Macrosep centrifugal concentrator, 3 K, Pall Filtron Co., Northborough, MA) at 5000*g* and 4 °C for 150 min. In a second step, the concentrated PG fractions were individually applied (~2 mL) to a gel filtration column (Hi Load 16/60 Superdex 75 Prep Grade, Amersham Biosciences), which was previously equilibrated with 50 mM sodium acetate buffer and 0.2 M NaCl, pH 4.4. Equilibration and elution (0.3 mL/min) in 50 mM sodium acetate buffer and 0.2 M NaCl were carried out at 25 °C. Fractions of 2 mL were collected, and the fractions showing PG activity were pooled and stored at 4 °C.

Pectinmethylesterase Activity Measurement. PME activity was determined by measuring the release of acid per time unit at pH 7.0 and 22 °C (*21*). The reaction mixture consisted of 200 μL of enzyme solution and 30 mL of a 0.35% apple pectin solution (70−75% esterification, Fluka) containing 125 mM 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 (Metrohm, Herisau, Switzerland). Every 15 s the consumption of 0.01 N NaOH was recorded during a 10 or 15 min reaction period. PME activity is proportional to the rate of consumption of NaOH ($\Delta V_{NaOH}/\Delta t$). PME activity can be expressed in units whereby 1 unit is defined as the amount of enzyme that produces 1 μmol of acid per minute at pH 7.0 and 22 °C.

Polygalacturonase Activity Measurement. PG activity measurement is based on the release of reducing groups (colorimetric method) (*22*). The purified enzyme solution (50 μL) was incubated with 300 μL of 0.2% polygalacturonic acid at 35 °C for 10 min. To stop the reaction, 2 mL of 0.1 M borate buffer, pH 9.0, and 400 μL of 1% cyanoacetamide were added to the reaction mixture and boiled for 10 min. After cooling, the absorbance was measured at 276 nm and 22 °C. Blank samples were determined in the same way without addition of enzyme. One PG unit was defined as the amount of enzyme producing 1 μmol of reducing groups per minute at 35 °C.

Gel Electrophoresis. The determination of molar mass and isoelectric point of the purified enzymes was performed in a Phastsystem (Amersham Biosciences). The molar mass was determined using SDS-PAGE in homogeneous media (20%). The proteins were silver stained. The molar mass was derived by comparison of the migration distance with a calibration curve of marker proteins (LMW marker: 14−97 kDa, Amersham Biosciences) with known molar mass: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). The isoelectric point (p*I*) was determined in PhastGel IEF media 3−9 (Amersham Biosciences). After migration, the proteins were silver stained. The marker proteins were amyloglucosidase (p*I* 3.50), soybean trypsin inhibitor (p*I* 4.55), β-lactoglobulin A (p*I* 5.20), bovine carbonic anhydrase B (p*I* 5.85), human carbonic anhydrase B (p*I* 6.55), myoglobin acidic band (p*I* 6.85), myoglobin basic band (p*I* 7.35), lentil lectin acidic band (p*I* 8.15), lentil lectin middle band (p*I* 8.45), lentil lectin basic band (p*I* 8.65), and trypsinogen (p*I* 9.30).

Isothermal Treatment. Isothermal inactivation experiments were performed in a water bath with temperature control. Purified tomato PG in 50 mM sodium acetate buffer, pH 4.4, was filled manually with a syringe in capillary tubes (200 μL, i.d. = 2 mm, 140 mm length, BlauBrand IntraMark, Wertheim, Germany). After preset time intervals at constant inactivation temperature, the samples were withdrawn and immediately cooled in ice−water, and the residual PG activity was measured within 2 h of storage in ice−water. No reactivation of PG was observed during storage.

High-Pressure Treatment. Purified tomato PG in 50 mM sodium acetate buffer, pH 4.4, was pressure treated in laboratory scale multivessel high-pressure equipment especially designed for kinetic experiments (HPIU-10.000, serial no. 95/1994, Resato, Roden, The Netherlands). The equipment is composed of eight individual vessels (volume = 8 mL, diameter = 10 mm, length = 100 mm) and permits a maximum pressurization of 1000 MPa in combination with temperatures ranging from −20 to 100 °C. The pressure medium was a glycol−oil mixture (TR15, Resato). A thermostated mantel, which surrounds each vessel, was connected to a cryostat to control the temperature during the experiment. The enzyme solutions were filled in flexible microtubes (0.3 mL, Elkay, Belgium) and were enclosed in the pressure vessels already equilibrated at a desired temperature. Pressure was built up slowly (~100 MPa/min) to minimize adiabatic heating. After the desired pressure had been attained, all individual vessels were isolated and the central circuit was decompressed. After a 2 min equilibration period to ensure isothermal conditions (*23*), one vessel was decompressed and the enzyme activity of this sample was considered as a blank (A_0). The other vessels were then decompressed after preset time intervals. After pressure release, the samples were immediately cooled in ice−water and the residual PG activity was measured within 2 h of storage in ice−water. No reactivation of PG was noticed during storage.

Kinetic Data Analysis. Inactivation kinetics of enzymes toward thermal and combined thermal/high-pressure processes usually follow a first-order kinetic model (eqs 1 and 2)

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

$$\log(A_t/A_0) = -t/D \tag{2}$$

where A_0 is the initial activity, A_t is the remaining activity at time t, k is the inactivation rate constant (min^{-1}), and D is the decimal reduction time (min). The inactivation rate constant k can be determined from a linear regression analysis of $\ln(A_t/A_0)$ versus treatment time at constant temperature, whereas the decimal reduction time D can be determined from a linear regression analysis of $\log(A_t/A_0)$ versus treatment time at constant temperature.

Using the Arrhenius model, the temperature dependence of the inactivation rate constant is expressed by the activation energy (E_a value):

$$\ln k = \ln k_{ref} + \frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right) \tag{3}$$

Using the thermal death time (TDT) model, the temperature dependence of the decimal reduction time is described by the z_T value:

$$\log D = \log D_{ref} - \frac{T - T_{ref}}{z_T} \tag{4}$$

The pressure dependence of the inactivation rate constant, expressed by the activation volume (V_a value), is determined on the basis of the Eyring equation:

$$\ln k = \ln k_{ref} - \left[\frac{V_a}{RT}(P - P_{ref})\right] \tag{5}$$

RESULTS AND DISCUSSION

Purification of Tomato Polygalacturonase. The crude PG extract was loaded on a cation exchange column (Hi Prep 16/10 SP/XL), and the elution profile presented a number of protein peaks at 280 nm. All fractions were analyzed for PME and PG activity. **Figures 1** and **2** present the elution profile in terms of UV absorption and PG and PME activity, respectively. The peak with the highest UV absorption displays PME activity (**Figure**

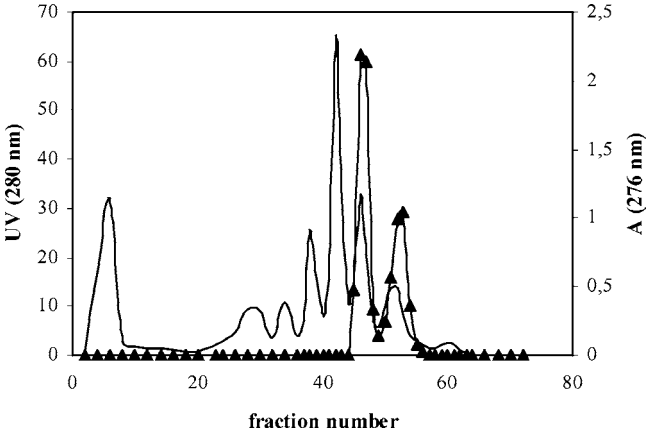

Figure 1. Elution profile for tomato PG crude extract on Hi Prep 16/10 SP/XL as a function of UV absorption and PG activity: UV (—); PG (▲).

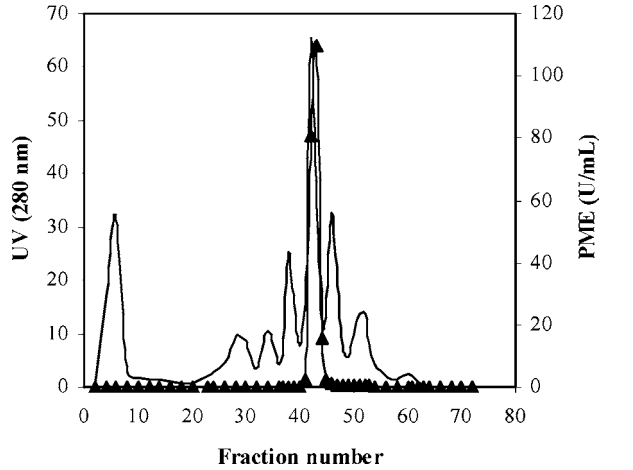

Figure 2. Elution profile for tomato PG crude extract on Hi Prep 16/10 SP/XL as a function of UV absorption and PME activity: UV (—); PME (▲).

2). As expected, two peaks showing PG activity were detected (**Figure 1**). The fractions presenting PG activity were collected and analyzed for thermostability. The PG collected from the first peak was completely inactivated after heating at 65 °C for 5 min, indicating the presence of PG2. The PG collected from the second peak was heat stable under the same heating conditions, confirming the presence of PG1. PG2 elutes first, followed by PG1, which is related to the basicity of the proteins (*20*). Because PG1 consists of an additional acidic subunit compared to PG2, PG1 will elute later. In a SDS-PAGE analysis of the fractions from the two peaks with PG activity, the lane that corresponds to PG1 presented one band, whereas the lane corresponding to PG2 presented multiple bands. The β-subunit is not detectable after silver staining (*4*). Pooled fractions of PG2 were applied on a gel filtration column (Hi Load 16/60 Superdex 75) (**Figure 3**). All fractions were tested for PG and PME activity. In none of the fractions was PME activity found. The fractions containing PG2 activity were pooled and stored at 4 °C for further use.

The estimated molar mass of purified tomato PG2 by SDS-PAGE is 42 kDa, which is in agreement with the literature. Purified tomato PG1 (without β-subunit) presented the same molar mass, confirming the data reported in the literature (*3, 5, 11, 20*). The isoelectric point for purified PG2 and PG1 was >9.3.

Thermal and Pressure Stability of Purified Tomato Polygalacturonase. The thermal stability of purified tomato

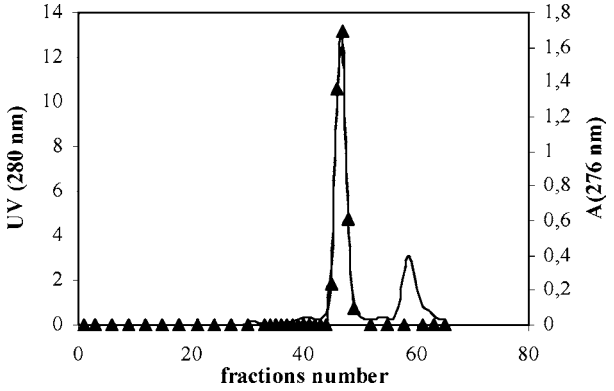

Figure 3. Elution profile for tomato PG2 on Hi Load 16/60 Superdex 75. Elution was performed with 50 mM sodium acetate buffer and 0.2 M NaCl, pH 4.4 (0.3 mL/min), at 25 °C. Fractions of 2 mL were collected: UV (—); PG (▲).

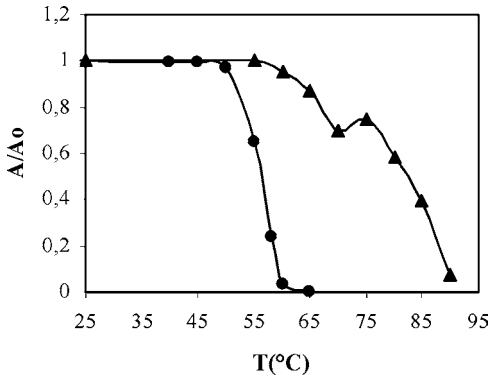

Figure 4. Thermal stability of purified tomato PG2 (●) and PG1 (▲) after 5 min at preset temperatures.

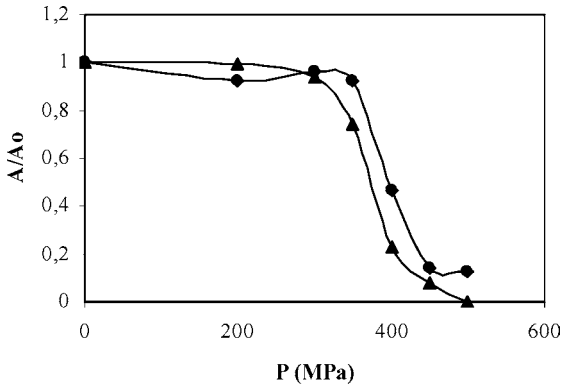

Figure 5. Pressure stability of purified tomato PG2 (●) and PG1 (▲) at 25 °C for 15 min.

PG2 and PG1 in 50 mM sodium acetate buffer, pH 4.4, was screened at preset temperatures using a treatment time of 5 min. **Figure 4** presents the relative residual activity as a function of temperature, indicating that purified tomato PG2 is a thermolabile enzyme, which is totally inactivated after 5 min at 65 °C. The heat stable enzyme (PG1) retains its activity after 5 min at 65 °C. Even after 5 min at 90 °C, there is still some residual PG activity.

Pressure stability of purified tomato PG2 and PG1 was screened by pressurizing samples for 15 min at 25 °C using different pressures (**Figure 5**). The reduction of the activity after 15 min is compared to the activity at time zero after the equilibration period (i.e., after 2 min). Up to 300 MPa there was almost no decrease in the activity of PG2 and PG1. At room temperature, PG2 and PG1 can both be inactivated in the

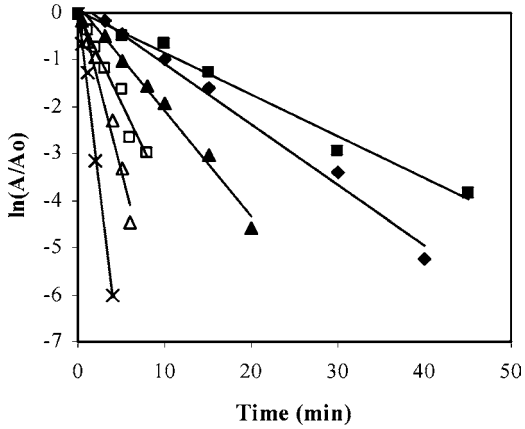

Figure 6. Isothermal inactivation kinetics of purified tomato PG2 in 50 mM sodium acetate buffer, pH 4.4: 53 °C (■); 55 °C (◆); 56.5 °C (▲); 58 °C (□); 60 °C (△); 63 °C (×).

Table 1. Estimated Kinetic Parameters for Isothermal Inactivation of Purified Tomato Polygalacturonase 2 in 50 mM Sodium Acetate Buffer, pH 4.4

T (°C)	k (min^{-1})	D (min)
53	0.089 ± 0.005[a]	25.85 ± 1.49
55	0.130 ± 0.006	17.66 ± 0.81
56.5	0.225 ± 0.010	10.21 ± 0.48
58	0.386 ± 0.030	5.95 ± 0.46
60	0.714 ± 0.049	3.22 ± 0.22
63	1.532 ± 0.048	1.50 ± 0.04
	E_a (kJ/mol)	z_T value (°C)
	270.6 ± 11.1	7.7 ± 0.3

[a] Standard error of regression.

pressure range from 300 to 500 MPa. For PG2 and PG1, there was no pressure stable fraction observed in the range studied. Although the thermostabilities of tomato PG2 and PG1 are clearly different, they present very similar pressure stabilities.

Isothermal Inactivation Kinetics of Purified Tomato Polygalacturonase 2. On the basis of the results of the thermal stability screening study, PG2 was thermally treated in the temperature range from 53 to 63 °C at atmospheric pressure. Inactivation kinetics could accurately be described by first-order kinetics confirmed by visual inspection of the model fitting and by the correlation coefficients of 0.971−0.997 obtained (**Figure 6**). The inactivation rate constants were estimated by linear regression analysis (*24*), and they vary from 0.089 min^{-1} at 53 °C to 1.532 min^{-1} at 63 °C (**Table 1**). The inactivation rate constants increase with increasing temperature. At 65 °C, PG2 was completely inactivated after 3 min of treatment, which is in agreement with existing literature data (*2*−*4*). The activation energy was determined by using the Arrhenius equation from the slope of the plot of the natural logarithm of the inactivation rate constants versus the reciprocal absolute temperature (K), and it was estimated as 270.6 kJ/mol ($r^2 = 0.933$).

As expected, the decimal reduction times decrease with increasing temperature, with an estimated z value of 7.7 °C. Lopez et al. (*16*) purified tomato PG2, and the heat inactivation (64−73 °C) also followed first-order kinetics, yielding a z value of 9.4 °C.

Combined Pressure−Temperature Inactivation of Purified Tomato Polygalacturonase 2. Combined pressure−temperature inactivation was studied in detail for purified tomato PG2. In ripe tomato fruit, PG2 is the main enzyme, as already

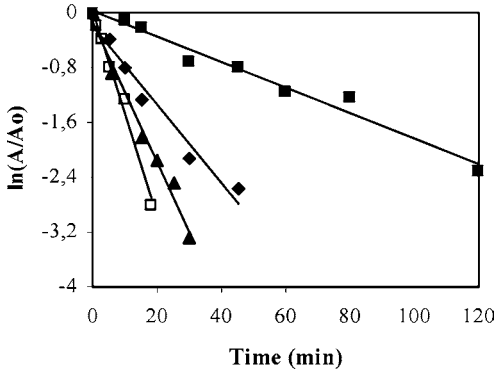

Figure 7. Isothermal/isobaric inactivation kinetics of purified tomato PG2 in 50 mM sodium acetate buffer, pH 4.4, at 20 °C: 350 MPa (■); 400 MPa (◆); 450 MPa (▲); 500 MPa (□).

observed during the purification procedure (see **Figure 1**), and no difference in pressure stability between PG1 and PG2 was observed. Combined treatments of pressure and temperature to inactivate purified tomato PG2 were carried out at 5−55 °C/100−600 MPa. The inactivation followed first-order kinetics, as visualized in **Figure 7** for different pressure levels at 20 °C. During pressure buildup, purified tomato PG2 is partially inactivated due to the temperature increase as a consequence of adiabatic heating. This activity loss varies from 10 to 70% depending on the pressure and temperature applied. The inactivation rate constants were determined from the slope of the natural logarithm of the relative residual activity versus treatment time for the temperature/pressure range studied (**Table 2**). From **Table 2**, a synergetic effect of pressure and temperature on the inactivation rate constants can be noted; that is, by increasing temperature at a fixed pressure, the inactivation rate constant increases. In the same way, by increasing pressure at a constant temperature, the inactivation rate constant also increases.

The pressure dependence of the inactivation rate constants was described by the Eyring equation, which was valid over the entire pressure/temperature range studied (**Table 3**). The activation volume was more or less constant at temperatures from 5 to 25 °C, followed by an increase in absolute value at 30 and 35 °C and again a decrease in absolute value at 50 °C. At all temperatures studied, the estimated activation volume was negative, indicating an acceleration of the inactivation reaction under pressure. The activation volume of tomato PG in crude extract followed the same trend (*18*), as well as lipoxygenase in green beans (25) and orange PME in crude extract (26). The pressure dependence of the inactivation rate constants of purified tomato PG2 was lowest at 15 °C.

The temperature dependence of the inactivation rate constants in the pressure range studied obeyed the Arrhenius equation (**Table 4**). The activation energy decreases at pressures up to 400 MPa, followed by an increase with further increasing pressure. A similar behavior was observed for *Bacillus subtilis* α-amylase (*27*), where at pressures up to 300 MPa the activation energy decreased with increasing pressure, whereas at higher pressures the estimated activation energy was approximately constant. For avocado polyphenol oxidase (*28*), an increasing pressure resulted in a decrease of the activation energy. The activation energy of purified tomato PG at atmospheric pressure was determined as 270.6 kJ/mol (see **Table 1**), which is not significantly different from the activation energy at 100 MPa. The influence of temperature on the PG inactivation rate is smallest at ∼400 MPa, as indicated by the lowest activation energy at this pressure.

Table 2. Estimated Inactivation Rate Constants ($\times 10^{-2}$ min^{-1}) for Isobaric/Isothermal Inactivation of Purified Tomato Polygalacturonase 2 in Sodium Acetate Buffer, pH 4.4

pressure (MPa)	temperature											
	5 (°C)	10 (°C)	15 (°C)	20 (°C)	25 (°C)	30 (°C)	35 (°C)	40 (°C)	45 (°C)	50 (°C)	53 (°C)	55 (°C)
100	nd[a]	nd	nd	nd	nd	nd	nd	nd	nd	2.88 ± 0.13	5.65 ± 0.25	13.92 ± 2.35[b]
200	nd	nd	nd	nd	nd	nd	nd	nd	1.92 ± 0.24	6.92 ± 0.50	8.10 ± 0.52	nd
300	nd	nd	nd	nd	nd	0.61 ± 0.04	1.42 ± 0.09	3.57 ± 0.19	6.30 ± 0.51	18.22 ± 2.76	nd	nd
350	nd	nd	nd	1.86 ± 0.12	3.81 ± 0.16	6.50 ± 0.46	7.69 ± 0.62	19.70 ± 2.93	nd	nd	nd	nd
400	nd	nd	3.76 ± 0.22	5.77 ± 0.56	8.56 ± 0.58	15.23 ± 1.34	22.27 ± 1.67	nd	nd	nd	nd	nd
450	nd	2.11 ± 0.16	4.43 ± 0.37	10.19 ± 0.65	16.81 ± 0.94	nd	nd	nd	nd	nd	nd	nd
500	1.89 ± 0.13	4.07 ± 0.36	9.49 ± 0.35	15.18 ± 0.94	nd	nd	nd	nd	nd	nd	nd	nd
550	2.67 ± 0.20	9.69 ± 0.97	14.37 ± 0.79	nd	nd	nd	nd	nd	nd	nd	nd	nd
600	6.09 ± 0.38	18.14 ± 0.85	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] Not determined. [b] Standard error of regression.

Table 3. Pressure Dependence of the Inactivation Rate Constants for Isothermal/Isobaric Inactivation of Purified Tomato Polygalacturonase 2 in 50 mM Sodium Acetate Buffer, pH 4.4

T (°C)	V_a (cm^3/mol)	r^2	T (°C)	V_a (cm^3/mol)	r^2
5	−27.04 ± 6.40[a]	0.996	25	−36.77 ± 1.92	0.997
10	−34.44 ± 1.50	0.947	30	−81.06 ± 22.01	0.931
15	−22.91 ± 3.71	0.950	35	−70.50 ± 9.24	0.983
20	−33.45 ± 5.82	0.943	50	−24.76 ± 0.71	0.999

[a] Standard error of regression.

Table 4. Temperature Dependence of the Inactivation Rate Constants for Isothermal/Isobaric Inactivation of Purified Tomato Polygalacturonase 2 in 50 mM Sodium Acetate Buffer, pH 4.4

P (MPa)	E_a (kJ/mol)	r^2	P (MPa)	E_a (kJ/mol)	r^2
100	270.9 ± 55.6[a]	0.960	400	66.7 ± 2.7	0.995
200	161.9 ± 45.5	0.927	450	99.1 ± 7.1	0.993
300	134.7 ± 6.3	0.993	500	96.2 ± 6.9	0.989
350	82.6 ± 9.6	0.961	550	112.4 ± 33.2	0.920

[a] Standard error of regression.

Inactivation of PG by pressure has already been reported in the literature. In diced tomato fruits almost no PG activity remained after a treatment at 800 MPa at 25 °C for 5 min (*29*), whereas for cherry tomatoes almost all PG activity was lost after treatments at 500 and 600 MPa for 20 min at room temperature (*30*). In tomato juice, Crelier et al. (*17*) reported a total inactivation of PG at 500 MPa after 20 min of treatment time at 30 °C.

On the basis of the estimated rate constants from isothermal as well as from isothermal/isobaric inactivation experiments (see **Tables 1** and **2**), a pressure−temperature kinetic diagram for purified tomato PG2 was constructed. Mathematical models to describe the dependence of the inactivation rate constants as a function of pressure and temperature have been developed using (i) the Arrhenius equation (*26*, *31*) or the Eyring equation (*32*) as a starting point, (ii) a modified thermodynamic equation (*18*, *25*, *33*, *34*), (iii) an elliptical equation (*35*), and (iv) models based on two parallel mechanisms of inactivation, each following first-order kinetics (*17*, *36*). In the present work, a modified thermodynamic equation (eq 6) was used to describe the

$$\ln(k) = \ln(k_0) - \frac{\Delta\alpha^{\ddagger}}{RT}(P - P_0)(T - T_0) - \frac{\Delta V_0^{\ddagger}}{RT}(P - P_0) + \frac{\Delta S_0^{\ddagger}}{RT}(T - T_0) \quad (6)$$

dependence of the inactivation rate constants for isothermal and isothermal/isobaric treatments. From eq 6, the estimated parameters determined by a nonlinear regression analysis (*24*) are presented in **Table 5**.

On the basis of the estimated parameters of eq 6, a simulated contour plot of inactivation rate constants as a function of pressure and temperature is depicted in **Figure 8**. The lines in the diagram represent combinations of pressure and temperature resulting in the same inactivation rate constant.

In conclusion, tomato polygalacturonase was purified by cation exchange chromatography yielding two distinct peaks with different heat stabilities. The PG2 fraction was further purified by gel filtration chromatography. Both enzymes (PG2 and PG1) showed the same molar mass (42 kDa) and an isoelectric point >9.3. Thermal inactivation of purified tomato PG2 at pH 4.4 followed first-order kinetics, and PG2 was

Table 5. Estimated Parameters for Pressure/Temperature Inactivation of Purified Tomato Polygalacturonase 2 in 50 mM Sodium Acetate Buffer, pH 4.4, at Reference Pressure of 400 MPa and Reference Temperature of 298 K

parameter	estimated value
$\Delta V_0^{\ddagger}$ (cm^3/mol)	-26.26 ± 5.60[a]
$\Delta S_0^{\ddagger}$ (J/mol·K)	217.39 ± 48.28
$\Delta\alpha^{\ddagger}$ (cm^3/mol·K)	0.581 ± 0.12
k_0 (min^{-1})	0.0419 ± 0.0068

[a] Standard error of regression.

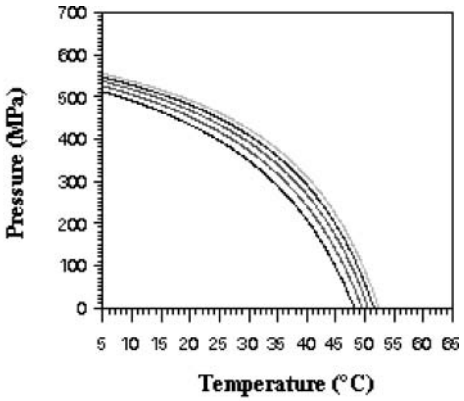

Figure 8. Simulated isorate contour plot for inactivation of purified tomato PG2 in 50 mM sodium acetate buffer, pH 4.4: (upper line) $k = 0.08$ min^{-1}; (lower line) $k = 0.04$ min^{-1}.

completely inactivated after 5 min at 65 °C. Pressure/temperature inactivation of purified tomato PG2 at pH 4.4 also followed first-order kinetics. A synergetic effect of pressure and temperature on the inactivation rate constants was noted. In addition, the thermostable fraction (PG1) shows a pressure stability very similar to that of PG2. Although it is still unclear whether PG1 exists in vivo or is to be considered as an artifact due to extraction, these results indicate that tomato PG2 (and PG1) can be inactivated by pressure without the need for high temperatures. A mathematical model used to describe the influence of temperature and pressure on the inactivation rate constants of purified tomato PG2 was formulated.

ABBREVIATIONS USED

PG, polygalacturonase; PME, pectinmethylesterase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IEF, isoelectric focusing; A_0, initial enzyme activity; A_t, enzyme activity at time t; D, decimal reduction time (min); D_{ref}, decimal reduction time at T_{ref} (min); E_a, activation energy (kJ/mol); k, first-order inactivation rate constant (min^{-1}); k_{ref}, first-order inactivation rate constant at T_{ref} or P_{ref} (min^{-1}); P, pressure (MPa); P_{ref}, reference pressure (MPa); R, universal gas constant (8.314 J/mol·K); t, time (min); T, temperature (°C or K); T_{ref}, reference temperature (°C or K); V_a, activation volume (cm^3/mol); z_T, z value (°C).

LITERATURE CITED

(1) Pressey, R.; Avants, J. K. Effect of substrate size on the activity of tomato polygalacturonase. *J. Food Sci*. **1971**, *36*, 486−489.
(2) Pressey, R.; Avants, J. K. Two forms of polygalacturonase in tomatoes. *Biochim. Biophys. Acta* **1973**, *309*, 363−369.
(3) Tucker, G. A.; Robertsons, N. G.; Grierson, D. The conversion of tomato-fruit polygalacturonase isoenzyme 2 into isoenzyme 1 in vitro. *Eur. J. Biochem*. **1981**, *115*, 87−90.
(4) Moore, T.; Bennett, A. B. Tomato fruit polygalacturonase isozyme 1. *Plant Physiol*. **1994**, *106*, 1461−1469.
(5) Ali, Z. M.; Brady, C. J. Purification and characterization of the polygalacturonases of tomato fruits. *Aust. J. Plant Physiol.* **1982**, *9*, 155−169.
(6) Pressey, R. Purification and characterization of tomato polygalacturonase convertor. *Eur. J. Biochem*. **1984**, *144*, 217−221.
(7) Watson, C. F.; Zheng, L.; DellaPenna, D. Reduction of tomato polygalacturonase β-subunit expression affects pectin solubilization and degradation during fruit ripening. *Plant Cell* **1994**, *6*, 1623−1634.
(8) Pressey, R. Changes in polygalacturonase isoenzymes and convertor in tomatoes during ripening. *HortScience* **1986**, *21*, 1183−1185.
(9) Pogson, B. J.; Brady, C. J. Do multiple forms of tomato fruit endopolygalacturonase exist in situ? *Postharvest Biol. Technol.* **1993**, *3*, 17−26.
(10) Tucker, G. A.; Robertsons, N. G.; Grierson, D. Changes in polygalacturonase isoenzymes during the ripening of normal and mutant tomato fruit. *Eur. J. Biochem*. **1980**, *112*, 119−124.
(11) Moshrefi, M.; Luh, B. S. Purification and characterization of two tomato polygalacturonase isoenzymes. *J. Food Biochem*. **1984**, *8*, 39−54.
(12) Eskin, M. N. A. Biochemical changes in raw foods: Fruits and vegetables. In *Biochemistry of Foods*, 2nd ed.; Academic Press: San Diego, CA, 1990; pp 124−129.
(13) Gould, W. A. *Tomato Production, Processing and Technology*, 3rd ed.; CTI Publications: Timonium MD, 1992.
(14) Knorr, D. Effects of high hydrostatic pressure processes on food safety and quality. *Food Technol*. **1993**, *June*, 156−161.
(15) Hendrickx, M.; Ludikhuyze, L.; Van den Broeck, I.; Weemaes, C. Effects of high pressure on enzymes related to food quality. *Trends in Food Sci. Technol.* **1998**, *9* (5), 197−203.
(16) Lopez, P.; Sanchez, A. C.; Vercet, A.; Burgos, J. Thermal resistance of tomato polygalacturonase and pectinmethylesterase at physiological pH. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 146−150.
(17) Crelier, S.; Robert, M. C.; Claude, J.; Juillerat, M. A. Tomato (*Lycopersicon esculentum*) pectin methylesterase and polygalacturonase behaviors regarding heat- and pressure-induced inactivation. *J. Agric. Food Chem*. **2001**, *49*, 5566−5575.
(18) Fachin, D.; Van Loey, A.; Indrawati; Ludikhuyze, L.; Hendrickx, M. Thermal and high-pressure inactivation of tomato polygalacturonase: a kinetic study. *J. Food Sci*. **2002**, *67*, 1610−1615.
(19) Pressey, R. Extraction and assay of tomato polygalacturonases. *HortScience* **1986**, *21*, 490−492.
(20) Pogson, B. J.; Brady, C. J.; Orr, G. R. On the occurrence and structure of subunits of endoplygalacturonase isoforms in mature-green and ripening tomato fruits. *Aust. J. Plant Physiol*. **1991**, *18*, 65−79.
(21) Crelier, S.; Tâche, M. C.; Raemy, A.; Renken, A.; Raetz, E. High-pressure for the inactivation of enzymes in food products: thermal and HHP treatment of tomato pectin methyl esterase. Poster presentation at the 9th World Congress on Food Science and Technology, Budapest, Hungry, 1995.
(22) Gross, K. C. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. *HortScience* **1982**, *17*, 933−934.
(23) Weemaes, C.; De Cordt, S. V.; Ludikhuyze, L. R.; Van den Broek, 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. Prog.* **1997**, *13*, 25−32.
(24) *SAS/STAT User's Guide*, version 6; SAS Institute: Cary, NC, 1994; pp 1135−1194.
(25) Indrawati; Van Loey, A. M.; Ludikhuyze, L. R.; Hendrickx, M. E. Lipoxygenase inactivation in green beans (*Phaseolus vulgaris* L.) due to high-pressure treatment at subzero and elevated temperatures. *J. Agric. Food Chem*. **2000**, *48*, 1850−1859.

(26) Van den Broeck, I.; Ludikhuyze, L.; Van Loey, A. M.; Weemaes, C. A.; Hendrickx, M. E. Inactivation of orange pectinesterase by combined high-pressure and temperature treatments: a kinetic study. *J. Agric. Food Chem*. **2000**, *48*, 1960−1970.

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

(28) Weemaes, C. A.; Ludikhuyze, L. R.; Van den Broek, I.; Hendrickx, M. E. Effect of pH on pressure and thermal inactivation of avocado polyphenoloxidase: a kinetic study. *J. Agric. Food Chem*. **1998**, *46*, 2785−2792.

(29) Shook, C. M.; Shellhammer, T. H.; Schwartz, S. J. Polygalacturonase, pectinesterase, and lipoxygenase activities in high-pressure-processed diced tomatoes. *J. Agric. Food Chem*. **2001**, *49*, 664−668.

(30) Tangwongchai, R.; Ledward, D. A.; Ames, J. M. Effect of high-pressure treatment on the texture of cherry tomato. *J. Agric. Food Chem*. **2000**, *48*, 1434−1441.

(31) Weemaes, C. A.; Ludikhuyze, L. R.; Van den Broeck, I.; Hendrickx, M. E. Kinetics of combined pressure temperature inactivation of avocado polyphenoloxidase. *Biotechnol. Bioeng.* **1998**, *60*, 292−300.

(32) Ludikhuyze, L.; Indrawati; Van den Broeck, I.; Weemaes, C.; Hendrickx, M. Effect of combined pressure and temperature on soybean lipoxygenase. 2. Modeling inactivation kinetics under static and dynamic conditions. *J. Agric. Food Chem*. **1998**, *46*, 4081−4086.

(33) Sonoike, K.; Setoyama, T.; Kuma, Y.; Kobayashi, S. Effect of pressure and temperature on the death rates of *Lactobacillus casei* and *Escherichia coli*. In *High Pressure and Biotechnology*; Balny, C., Hayashi, R., Heremans, K., Masson, P., Eds.; Coloque INSERM/John Libbey Eurotext: Montrouge, Frace, 1992; Vol. 224, pp 297−301.

(34) Indrawati; Van Loey, A. M.; Ludikhuyze, L. R.; Hendrickx, M. E. Soybean lipoxygenase inactivation by pressure at subzero and elevated temperatures. *J. Agric. Food Chem*. **1999**, *47*, 2468−2474.

(35) Reyns, K. M. F. A.; Soontjes, C. C. F.; Cornelis, K.; Weemaes, C. A.; Hendrickx, M. E.; Michiels, C. W. Kinetic analysis and modelling of combined high-pressure−temperature inactivation of the yeast *Zygosaccharomyces bailii*. *Int. J. Food Microbiol.* **2000**, *56*, 199−210.

(36) Stoforos, N. G.; Crelier, S.; Robert, M. C.; Taoukis, P. S. Kinetics of tomato pectinmethylesterase inactivation by temperature and high pressure. *J. Food Sci*. **2002**, *56*, 1404−1407.

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