Tomato (*Lycopersicon esculentum*) Pectin Methylesterase and Polygalacturonase Behaviors Regarding Heat- and Pressure-Induced Inactivation

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The combined high pressure/thermal (HP/T) inactivation of tomato pectin methyl esterase (PME) and polygalacturonase (PG) was investigated as a possible alternative to thermal processing classically used for enzyme inactivation. The temperature and pressure ranges tested were from 60 °C to 105 °C, and from 0.1 to 800 MPa, respectively. PME, a heat-labile enzyme at ambient pressure, is dramatically stabilized against thermal denaturation at pressures above atmospheric and up to 500−600 MPa. PG, however, is very resistant to thermal denaturation at 0.1 MPa, but quickly and easily inactivated by combinations of moderate temperatures and pressures. Selective inactivation of either PME or PG was achieved by choosing proper combinations of *P* and *T*. The inactivation kinetics of these enzymes was measured and described mathematically over the investigated portion of the *P*/*T* plane. Whereas medium composition and salinity had little influence on the inactivation rates, PME was found less sensitive to both heat and pressure when pH was raised above its physiological value. PG, on the other hand, became more labile at higher pH values. The results are discussed in terms of isoenzymes and other physicochemical features of PME and PG.

Keywords: *tomato; Lycopersicon esculentum; pectin methylesterase; polygalacturonase; inactivation; kinetics; high pressure; temperature; stabilization; selective inactivation*

INTRODUCTION

Retention of viscosity is one of the most important quality criteria in the tomato processing industry (*1*). The ability of a puree, juice, or concentrate to hold its solid portion in suspension over the shelf life of the product is mainly dependent upon the total amount and quality of pectic material present in the system (*2, 3*). Viscosity loss in tomato-based products is the direct consequence of the degradation of pectic substances by endogenous enzymes, i.e., pectin methylesterase (PME, E. C. 3.1.1.11) and endo-polygalacturonase (PG, E.C. 3.2.1.15). PME catalyses the hydrolytic cleavage of the methylester moieties on pectin molecules, resulting in the release of methanol and partially de-esterified pectin. This process per se has no direct impact on the medium viscosity. However, the lower the degree of esterification (DE) of the pectin molecule, the better substrate it becomes for PG (*4, 5*). On one hand, PME enhances the action of PG, which hydrolyses the α-1,4-glycosidic bonds of polygalacturonic acid chains (*5*). On the other hand, it is well-known that pectin may, in the demethylated form, bind Ca^{2+} ions to form calcium pectate, which not only forms a gel network but also shows an increased resistance to polygalacturonase attack and would thus help preserve if not increase the product texture (*6*).

Surprisingly, little attention has been brought to the inactivation of PG in tomato products as compared to the large amount of literature published on the behavior of PME under thermal or hyperbaric denaturing conditions. This is all the more astonishing considering that PG-induced breakdown of the pectin molecules is the main cause for texture/viscosity loss, whereas PME "only" leads to cloud reduction in juices.

Thus, an optimal treatment should completely inactivate PG while preserving PME activity. However, this result cannot be achieved with the thermal processing traditionally used to inactivate pectic enzymes. At present, a thermal treatment, if necessary followed by partial removal of excess juice through centrifugal decantation, helps minimize the enzymatic breakdown of pectin in tomato products (*7*−*9*). PG is a very heat-resistant enzyme though, and combinations of high temperatures and long treatment times (e.g., 90−95 °C/30 min) are required to reduce its activity to a satisfactory level. This treatment will then quickly and totally destroy the PME activity, which is much more heat-labile. This process is known as "hot-break" (*1*) and takes place in the earliest steps of tomato processing. The major drawback of the thermal treatments lies in the negative impact generally observed on the treated product color, flavor, taste, and nutritional value due to heat-induced degradation processes (*1, 10*).

In this context, high hydrostatic pressure treatment (HHPT) could be a valuable alternative to the heating of tomato products. High pressure, combined if necessary with moderate heating, has shown in many studies its ability to destroy microbial contaminants (*11*−*13*) and inactivate enzymes (*14*) while preserving fresh product characteristics. In addition pressure, unlike

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temperature, is transmitted instantaneously and uniformly throughout the sample (Pascal's principle), making its impact independent of sample size and geometry (*15*). Also, it responds to a growing demand for high quality products that are fresh-like, minimally processed and additive-free. Due to a slow development in western countries, only a few industrial applications of HHP technology have appeared on the market in recent years (*16*). This situation is probably due in part to the lack of properly documented inactivation kinetics for food quality-related enzymes and microorganisms, a key information in the context of industrial implementation for high-pressure technology (*14*, *17*). For a more detailed overview on the subject, the reader is referred to such papers as those in refs 18−20.

In the present study, the impact of combined HP/T treatments on tomato PME and PG was investigated and compared with the heat-induced inactivation of the same enzymes at atmospheric pressure. Tomato juice, liquid tomato extracts, as well as buffered enzyme solutions were treated in order to evaluate the effect of the surrounding medium on the inactivation process. A mathematical model was used to describe the behavior of PME and PG over the investigated range of pressure and temperature.

On-line, in situ measurement techniques under hyperbaric conditions such as the diamond anvil cell described in (*21*), the stopped-flow apparatus described in (*22*) or the HP/HT bioreactor featured in (*23*) are extremely valuable sources of information regarding the changes occurring during high-pressure trials. Unfortunately, they are only suited for optically transparent media and/or purified enzymes. Hence, they could not be applied to the present system. The main target of the present study was, however, to gain more insight into the overall inactivation effects that could be obtained with the selected HP/T combinations.

MATERIALS AND METHODS

Sample Preparation. Fully ripe tomatoes (*Lycopersicon esculentum*) of the variety Nema 1401 were obtained from the Nestlé R&D Center, Badajoz (Spain). They had a 5.87 ± 0.11% total- and a 4.25 ± 0.09% soluble solid content, as measured with a Mettler PM100 weighing scale combined with a LP-16 infrared oven (Mettler Toledo AG, Greifensee-CH). The tomatoes were washed and wiped dry prior to being crushed in a household blender. The seeds and the largest pieces of tomato skin were removed by passing the resulting juice through a kitchen strainer (mesh size 1 × 1 mm). The mixture was then homogenized, and the size of the solid particles further reduced using a benchtop Polytron PT 3000 with a 30 mm head (Kinematica AG, Littau-CH) at 10 000 min^{-1} for 2 min. Finally, the crushed tomatoes were degassed under vacuum. All these preparation steps were performed at 4 °C.

Samples of juice (ca. 10−15 mL) were then transferred into heat-sealed polyethylene pouches. These offered an interesting surface/volume ratio ensuring a quick transfer of heat to and from the sample. All of the material was kept on ice until they were processed. All samples were duplicated and processed together.

Tomato PME and PG interact with cell walls components by ionic forces. In the tomatoes that were used, less than 10% of the total PME activity was measured in the supernatant collected after the 10 min centrifugation at 20 000 g/4 °C of a tomato juice sample. However, the enzyme can be easily desorbed by increasing both pH and ionic strength (*24*). To evaluate a possible stabilizing effect of the insoluble matrix, PME-rich liquid extracts were hence produced. The pH of the juice was adjusted to 7.0 (with 2N NaOH), solid NaCl was added to a final concentration of 0.5 M, and the mixture was centrifuged 20 min at 3 000 g. The clear supernatant was then collected and its pH brought back to the juice initial value of 4.2 (with 2N HCl). To further identify a possible influence of cosolutes in the liquid fraction of the tomato juice, an aqueous solution of a purified tomato PME (Product P 6763, Lot # 23H8055 from Sigma Chemie, Buchs, CH) was also used in some experiments. The enzyme activity in the solution was adjusted to the same level than in tomato juice.

Thermal Inactivation Treatments. Tomato PME was inactivated in the samples at 60 °C, 65 °C, 70 °C, and 75 °C, and the residual enzyme activity measured as a function of treatment time. Tomato PG was, due to its higher thermal resistance, treated between 80 and 105 °C. 2.0−2.5 mL samples of tomato juice were transferred with a syringe into a coil of stainless steel tubing (2 mm internal diameter, 0.5 mm thick, 750 mm long), which ensured a very quick heating and cooling of the juice within. Both extremities of the coil were screwed together tightly, and the sample was kept in an ice−water bath until treated. At time zero (T_{sample} = 0 °C), the coil was dropped into a water bath set at the appropriate temperature and kept there for the desired time before it was taken out and immediately dipped into the ice−water bath for cooling. Residual enzyme activity was then measured in the sample.

High-Pressure Treatments. The HHP treatments were performed using a single vessel ABB QFP-6 unit (ABB, Västerås, Sweden). The 1 L high pressure chamber was 90 mm in diameter and had a 225 mm usable height (d = 85 mm and h = 190 mm if using a loading basket). The machine used water as pressure-transmitting medium, and was equipped with a heating jacket for temperature control. Pressure was varied between 0 and 800 MPa, for working temperatures between 30 °C and 80 °C. The rate of pressure build-up was approximately 300 MPa/min and holding times ranged from 1 to 60 min. The equipment was fitted with pressure- and temperature-monitoring devices.

The temperature T_0 of the pressure-transmitting liquid in the chamber was adjusted just before pressurization. The samples were then loaded and immediately treated. Taking the adiabatic heat of compression into account when setting T_0, the desired combination of pressure and temperature could thus be obtained directly at the end of the pressure build-up phase.

After pressurization, the tomato samples were stored on ice until their residual enzyme activity was measured. When this was not possible directly after treatment, the samples were frozen at −25 °C and analyzed later on.

Time zero corresponds to a sample that was brought to the desired P/T combination and immediately depressurized. It was observed that PME and PG activity in these samples did not differ significantly from untreated ones.

Activity Measurements. *Pectin Methyl Esterase (PME).* Among the various assays that were developed for PME, which were reviewed for example by (*25*), the pH-stat technique is the most widely used. It consists of the on-line titration of the carboxylic groups generated by PME during the hydrolysis of a pectin solution. All measurements were performed at 21 °C.

A 21 mL portion of a pH 7, 0.5% aqueous suspension of apple pectin (70−76% methylated, USP-grade from Sigma Chemie, Buchs, CH) was mixed with 7.5 mL of a 0.5 M NaCl solution in a titration vessel. The volume was adjusted to 30 mL with distilled water and the tomato juice sample (typically 1.4 mL water and 100 μL sample). The 200 μL pipet tips that were used had to be cut at 6−8 mm above the tip, allowing a more "representative" sampling of the rather thick tomato juice. The pH was adjusted to 7.5 and maintained at this value during hydrolysis by use of a titration unit consisting in a Metrohm 691 pH meter, 614 Impulsomat and 665 Dosimat (Metrohm AG, CH-9100 Herisau). The volume V_{NaOH} of base (0.01 N sodium hydroxide) was then monitored as a function of time on a Philips PM 8262 X/t recorder. All samples were measured in triplicate.

The slope $S = dV_{NaOH}/dt$ was determined in the linear part of the titration curve. It is directly proportional to A_{sp}, the

Table 1. First Order Inactivation Rate Constants k (s^{-1}) measured for the Thermal Inactivation of PG in a Tomato Juice As a Function of Temperature (°C)

T (°C)	80	85	85b	90	97	105
k (s^{-1})	$4.52 \times 10^{-4} \pm 2.13 \times 10^{-5}$[a]	$1.27 \times 10^{-3} \pm 8.20 \times 10^{-5}$	$1.12 \times 10^{-3} \pm 1.07 \times 10^{-4}$	$1.92 \times 10^{-3} \pm 2.53 \times 10^{-4}$	$3.09 \times 10^{-3} \pm 6.51 \times 10^{-4}$	$1.31 \times 10^{-2} \pm 9.03 \times 10^{-4}$

activity per mL of PME-containing sample, which is obtained as follows (eq 1):

$$A_{sp}(\mu\text{equH}^{+}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}) = S(\text{mL}\cdot\text{min}^{-1})\cdot\frac{N_{NaOH}(\mu\text{mol}\cdot\text{mL}^{-1})}{V_{sample}(\text{mL})} \quad (1)$$

At pH 7.5 the pK_a of the carboxylic groups does not have to be taken into account and there is a direct relation between the amounts of sodium hydroxide injected in the medium and the μmole of [$-COO^-$] groups released.

Residual PME activity A_{rel} was then plotted as a function of processing time for every P/T combination.

Polygalacturonase (PG). PG was assayed by measuring the appearance of reducing groups accompanying the cleavage of a polygalacturonic acid substrate (sodium polypectate from Sigma Chemie, Buchs, CH).

Instead of time-consuming dialysis steps, an adaptation of the procedure described in (*26*) was used prior to the dosage of PG in order to remove the tomato endogenous sugars: The tomato juice sample (100 μL) was centrifuged (20 000*g*, 4 °C, 10 min), the supernatant discarded and the pellet re-suspended in 100 μL ice-cold distilled water. The tomato PG, a membrane-bound enzyme, was not solubilized at pH 4.2 (tomato juice natural pH) and such low ionic strength conditions, and remained in the pellet. After three washing cycles, the pellet was re-suspended in a 50 mM, pH 4.8 succinic acid buffer up to a volume of 70 μL.

The samples prepared as described above and the substrate solution (2% polygalacturonic acid in 50 mM, pH 4.8 succinate buffer) were preheated at 45 °C. The assay was started by adding 630 μL of substrate solution to the sample and continuously shaking while maintaining the temperature at 45 °C.

100 μL samples were taken at times 0, 20, 40, and 60 min and the reaction immediately stopped by mixing them with an equal amount of 0.3 M NaOH and storing them on ice. The samples were then centrifuged 2 min at 20 000*g*/4 °C.

Reducing sugars were detected using 3,5-dinitrosalicylic acid (DNSA), from Merck. The reagent solution was prepared by dissolving 1 g DNSA in 20 mL NaOH 2N before adding 30 g potassium sodium tartrate (tetrahydrate form, Merck) and completing the volume to 100 mL. Equal volumes of sample supernatant and reagent solution were mixed, heated for 15 min at 100 °C in a heating block, diluted 10 times with dist. H_2O and finally centrifuged (20 000*g*, 2 min). The absorbency of the supernatant was then measured at 540 nm. The reading was transformed into an equivalent concentration of galacturonic acid using a calibration curve. Each tomato sample was measured in triplicate.

RESULTS AND DISCUSSION

PME and PG Thermal Inactivation. The inactivation curves $A_{rel} = f(t)$ were treated according to a first-order inactivation model described in eq 2. The latter can be linearized by plotting $\ln(A_{rel})$ vs t, as shown in Figure 1 for the tomato juice enzyme. The first-order inactivation rate constant k [s^{-1}] was then obtained from the slope of each straight line

$$A_{rel} = \frac{A(t)}{A_0} = \exp(-k\cdot t) \quad (2)$$

The data plots showing PG residual activity as a function of heating time are shown in Figure 2. Table 1 lists the corresponding values of the first-order inactivation rate constant, k. All the k values obtained for both PMEs and for PG at 0.1 MPa were reported on the Arrhenius plot shown in Figure 3, which clearly demonstrates the much higher heat resistance of PG. Its inactivation is slower than that of PME at all investigated temperatures and is less strongly influenced by this parameter, which translates into an activation energy E_a of 134.5 ± 15.7 kJ·mol^{-1}.

In the case of PME, the activation energies calculated for the inactivation of the purified (Sigma tomato PME) and crude tomato juice enzymes were respectively 267.6 ± 22.1 and 350.1 ± 6.0 kJ·mol^{-1}. For the thermal inactivation of a commercial tomato PME in distilled water, the authors of ref 27 obtained 324.1 ± 24.1 kJ·mol^{-1}, which is in fair agreement with our finding.

Figure 3 also shows that the inactivation rates measured for the purified and the tomato juice enzymes

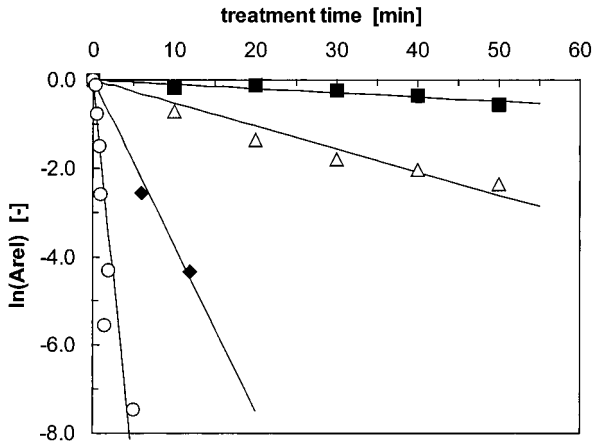

Figure 1. Thermal inactivation (0.1 MPa) of PME in a tomato juice at (■) 60 °C, (△) 65 °C, (◆) 70 °C, and (○) 75 °C. Straight lines correspond to the linearization of the first-order inactivation model.

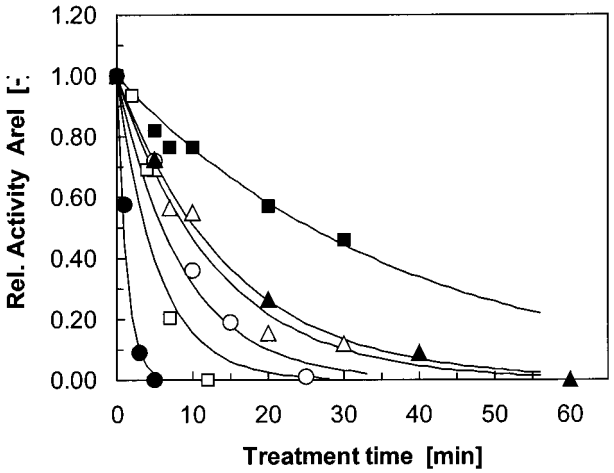

Figure 2. Thermal inactivation (0.1 MPa) of PG in a tomato juice at (■) 80 °C, (△) 85 °C, (▲) 85 °C b, (○) 90 °C, (□) 97 °C, and (○) 105 °C. Curves were calculated using the corresponding values of k (first-order inactivation rate constant) listed in Table 1.

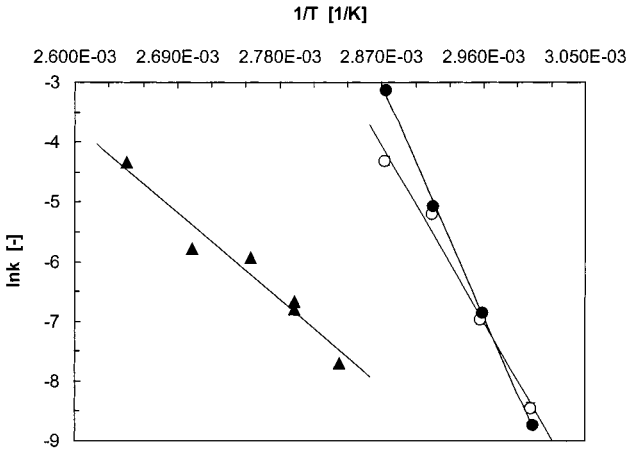

Figure 3. Arrhenius plot for the thermal inactivation of (○) purified tomato PME, (●) tomato juice PME, and (▲) tomato juice PG. Straight lines correspond to the Arrhenius model.

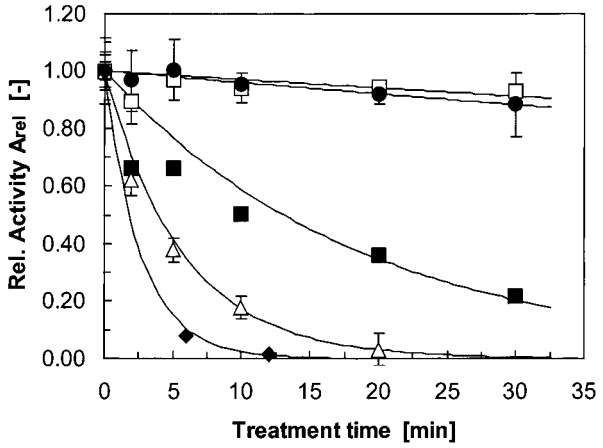

Figure 4. Inactivation of tomato juice PME at 70 °C and (◆) 0.1 MPa, (■) 100 MPa, (□) 300 MPa, (●) 600 MPa, (△) 800 MPa.

are too similar to allow any conclusion regarding the impact of medium composition on the inactivation rate. In fact, the observed disparity could be caused by differences in tomato variety, fruit maturity or isolation protocol (in the case of the purified material).

Several authors have, on the other hand, identified a protective effect of the solid matrix and other cosolutes present in the medium (*28, 29*). For instance, Tajchakavit and Ramaswamy (*30*) found that PME in orange juice was stabilized against heat denaturation by increases in both pH value and total solid content.

Upon studying the thermal resistance of raw or purified tomato PG and PME at physiological pH, the authors in ref 31 also observed that a given enzyme in a "thinner" environment was more heat-labile.

The thermal inactivation of PME in various fruit and vegetable model systems has been the object of many accounts in the scientific literature, very often in terms of D (decimation time) and z (ΔT corresponding to a 90% decrease of D) parameters. The data obtained by various authors (*27, 31*−*35*) were compared, and all the obtained values of the inactivation rate constant k reported along with our measurements on the same Arrhenius plot (data not shown). Despite widely different model systems and treatment conditions, it clearly appears that the inactivation rate of PME is more heat-sensitive at lower temperatures. Roughly in the 55−70 °C domain, E_a values above 300 kJ·mol^{-1} can be evaluated, whereas they drop down to about 100 kJ·mol^{-1} between 70 and 95 °C. This behavior could be partially explained by the coexistence of a heat-sensitive PME isoenzyme which inactivation is measured in the low-temperature range, and a heat-resistant isoform. This observation is not the mere result of different model systems or enzyme origins, as such a "biphasic Arrhenius plot" can be observed for two model systems which were investigated over a large temperature domain (*33, 34*). It is also reported in ref 33 that PME (from various tomato species) is more stable at higher pH values.

Earlier experiments had shown that tomato PME could be partially inactivated at 800 MPa and a mild temperature of 30 °C (*36*). The same inactivation levels were obtained for identical, total pressurization times, independently of the number of pressure cycles (1 × 30 min, 3 × 10 min or 4 × 7.5 min), which indicated a limited impact of the pressure build-up and release phases on the enzyme activity. In the case of a purified *B. subtilis* α-amylase (15 mg·mL^{-1} in 10 mM Tris HCl, pH 8.6) though, the authors of ref 37 found that the treatment impact was slightly increased when total pressurization time was divided into four to five treatment cycles. The observed effect leveled off upon further division.

This type of high pressure treatment only allows the observation of effects which can be measured after the system has been brought back to ambient pressure and temperature (i.e. slowly reversible or irreversible effects). Reversible denaturation of proteins is usually observed below 300 MPa, but examples of renaturation even after treatments at higher pressures have been given in ref 38 for Taka-amylase A (60−950 MPa range) or ref 39 for metmyoglobin (750 MPa). It was checked several times during the present investigation that the measured changes in enzymatic activity were indeed of the irreversible type (data not shown). This was achieved by measuring residual enzyme activity in the same pressurized samples at regular time intervals.

Inactivation of PME under Hyperbaric Conditions. Figure 4 shows the inactivation curves for tomato juice PME at 70 °C and pressures varying between 100 and 800 MPa. The inactivation at atmospheric pressure (0.1 MPa) was added for reference. The graph shows also that whereas PME is quickly inactivated at 0.1 MPa/70 °C, the inactivation rate constant k dramatically decreases as soon as pressure is raised. It reaches a minimum between 300 and 600 MPa, and then starts to increase again. However, even at 800 MPa, k is still inferior to its corresponding value at atmospheric conditions.

These counteracting effects of pressure and temperature were observed at all other investigated temperatures, i.e., 75 °C, 65 °C, and 60 °C. However, the pressure corresponding to maximal stabilization effect (i.e., minimal value of k) seemed to shift to lower values as T was decreased. Below 70 °C for instance, the inactivation measured at 800 MPa became again faster than the one at 0.1 MPa. This is in agreement with previous measurements made at lower temperatures, i.e., 30 °C or 45 °C (*36*). At such values, the enzyme is unaffected at 0.1 MPa, but can be partially inactivated under hyperbaric conditions (data not shown).

Table 2 displays the values of k for PME inactivation, which correspond to the various P/T combinations that were investigated.

Table 2. First Order Inactivation Rate Constants k (s^{-1}) Measured for Tomato Juice PME as a Function of Pressure (MPa) and Temperature (°C)

	1st order inactivation rate constant k (s^{-1})			
P (MPa)	60 °C	65 °C	70 °C	75 °C
0.1	$1.61 \times 10^{-04} \pm 7.98 \times 10^{-6}$[a]	$8.72 \times 10^{-04} \pm 7.34 \times 10^{-5}$	$6.25 \times 10^{-03} \pm 6.80 \times 10^{-4}$	$3.68 \times 10^{-02} \pm 1.58 \times 10^{-3}$
100	$7.58 \times 10^{-06} \pm 9.08 \times 10^{-7}$	$1.63 \times 10^{-04} \pm 1.47 \times 10^{-5}$	$8.83 \times 10^{-04} \pm 7.95 \times 10^{-5}$	$5.74 \times 10^{-04} \pm 3.56 \times 10^{-5}$
200	ND	ND	ND	$1.37 \times 10^{-04} \pm 1.10 \times 10^{-5}$
300	$9.70 \times 10^{-06} \pm 1.26 \times 10^{-6}$	$1.12 \times 10^{-04} \pm 1.54 \times 10^{-5}$	$5.14 \times 10^{-05} \pm 4.78 \times 10^{-6}$	$1.83 \times 10^{-05} \pm 1.48 \times 10^{-6}$
400	ND[b]	ND	ND	$3.86 \times 10^{-05} \pm 2.70 \times 10^{-6}$
500	ND	ND	ND	$1.03 \times 10^{-04} \pm 9.07 \times 10^{-6}$
600	$4.38 \times 10^{-05} \pm 5.26 \times 10^{-6}$	$4.43 \times 10^{-05} \pm 3.90 \times 10^{-6}$	$7.02 \times 10^{-05} \pm 7.23 \times 10^{-6}$	$5.25 \times 10^{-05} \pm 3.57 \times 10^{-6}$
700	ND	ND	ND	$3.02 \times 10^{-04} \pm 2.20 \times 10^{-5}$
800	$1.50 \times 10^{-03} \pm 9.27 \times 10^{-5}$	$2.33 \times 10^{-03} \pm 1.98 \times 10^{-4}$	$2.92 \times 10^{-03} \pm 1.69 \times 10^{-4}$	$2.71 \times 10^{-03} \pm 2.49 \times 10^{-4}$

[a] Standard errors are given. [b] ND = not determined.

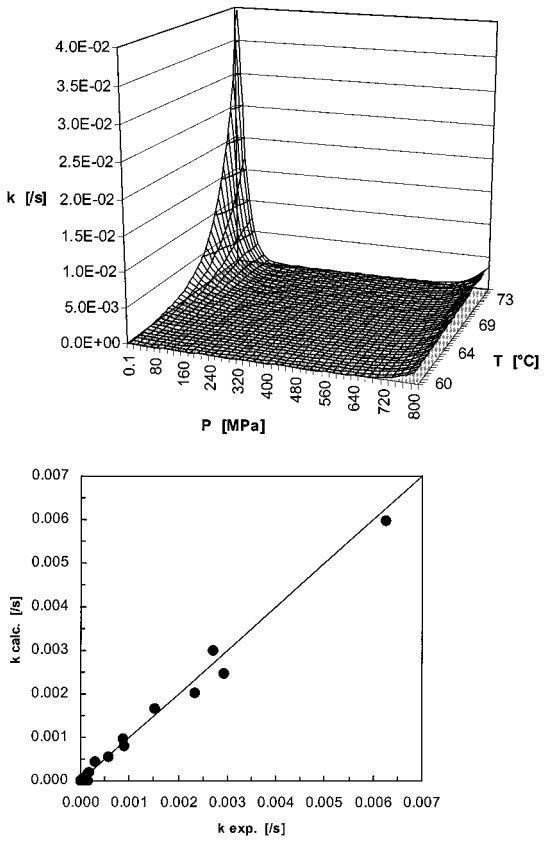

Figure 5. Evolution of the first-order inactivation rate constant k for tomato PME in a juice over the investigated P/T ranges (eq 3−6). Insert graph: Calculated vs measured values of k.

Due to the nonlinear evolution of ln(k) as a function of pressure, Eyring equation could not be used to describe the obtained results. An empirical model, equations,[3−7] was then developed to take into account the influence of P and T on the inactivation rate constant k. Figure 5 shows the corresponding surface in 3D view. The insert graph shows the quality of the fit obtained with equations[3−7] by plotting the predicted values of k against the measured ones. The corresponding correlation coefficient r^2 was 0.9902

$$k(P) = k_o \cdot (\exp(-A \cdot P)) + \exp(B \cdot (P - C)) \quad (3)$$

We determined a value of k_o, A, B, and C for each investigated temperature. Equations 4−6 were then developed to describe the evolution of these four parameters with T.

$$k_o(T) = 3.067 \times 10^{-12} \cdot \exp(3.641 \times 10^{-1} \cdot T) \quad (4)$$

$$A(T) = 3.681 \times 10^{-4} \cdot T^2 - 4.898 \times 10^{-2} \cdot T \cdot + 1.645 \quad (5)$$

$$B(T) = \text{const.} = 1.900 \times 10^{-2} \quad (6)$$

$$C(T) = 1.714 \times 10^{1} \cdot \text{T} - 3.531 \times 10^{-2} \quad (7)$$

Although not investigated in so much detail, the purified tomato PME from Sigma and the enzyme in thin extracts at pH 4.2 behaved in a very similar way and showed the same trends regarding stabilization by pressure against denaturing temperatures (data not shown).

Many authors have investigated the impact of high-pressure treatments on PME activity in products such as tomato or citrus juice (e.g. *10, 29, 40*). Most of these papers show that drastic treatment conditions are usually required to inactivate PME under hyperbaric conditions. Counteracting effects of pressure and temperature were also identified in these earlier studies for tomato PME, and a description of the phenomenon which corroborates the present findings is given in (*27*). It should be noted on the other hand that in the case of (purified) orange PME, pressure and temperature acted in synergy (*41*).

A distinction has to be made at this point between protein/enzyme stabilization and activation by pressure. The former was observed above for PME, and the latter has been reported for instance in the case of pear fruit polyphenoloxidase (*42, 43*). Both are linked to pressure-induced conformational changes of the enzyme molecules. On the other hand, for cellulases (*44*) and proteases (*45*), the acceleration of the digestion process seemed to be due mainly to pressure-induced changes in the substrates conformation, which made them more prone to enzymatic degradation. Due to the number of steps involved in enzymatic processes, it is usually difficult to clearly identify which particular steps or compounds were most affected by pressure (*46*), all the more when substrate is present in the pressurized medium (*27, 46*). This requires a different approach/methodology, but there is nevertheless a growing amount of evidence suggesting that true activation might be achieved through P-induced conformational changes of an enzyme such as α-chymotrypsin (*47*).

The influence of medium composition and pH was investigated again for tomato PME, but under hyperbaric conditions. Experiments were performed at two different pressures (300 and 800 MPa) and three temperatures (60 °C, 65 °C, and 70 °C). Three types of

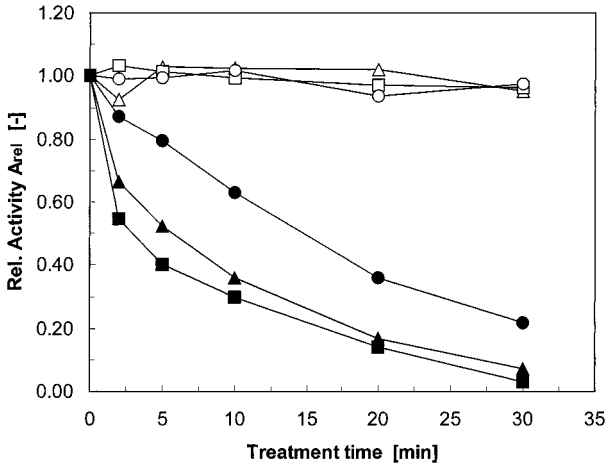

Figure 6. Residual PME activity in samples of various complexity and pH pressurized at 60 °C. (△) 300 MPaA/TJ, (□) 300 MPa/TX4, (○) 300 MPa/TX7, (▲) 800 MPa/TJ, (■) 800 MPa/TX4, (●) 800 MPa/TX7.

samples were processed: Tomato juice at natural pH = 4.2 (labeled "TJ4" in Figure 6), liquid tomato extract, pH 4.2, 0.5 M NaCl ("TX4") and liquid tomato extract, pH 7.0, 0.5 M NaCl ("TX7"). Figure 6 shows the results that were obtained at 60 °C, but the same trends were observed at 65 and 70 °C: The enzyme is completely barostable in all media at 300 MPa, and no difference in the behavior were hence noticed at this pressure. The removal of the juice solid fraction and the addition of 0.5 M NaCl had no effect on the inactivation pattern. Increasing pH to 7.0, on the other hand, even stabilizes the enzyme against pressure-induced denaturation. In ref 48 for example, upon measuring the high-pressure inactivation of PME in orange juice, it was also observed that the enzyme was stabilized against denaturation by increased pH values (as well as solid content).

The pressure-induced stabilization of proteins against heat denaturation is not something new and has been described for a variety of model systems, some of which have been reviewed by Mozhaev et al. (*47*). The authors of ref 23 studied two different β-glucosidases. They observed counteracting effects of P and T in the case of the almond enzyme, and activation under hyperbaric conditions for the β-glucosidase of *S. solfataricus*. The authors of refs 28 and 49 also observed a pressure-induced stabilization against heat denaturation with three different β-galactosidases (*A. oryzae, K. lactis, and E. coli*). The case of tomato PME seems however to be one of the most spectacular encountered so far.

This type of behavior can be explained by the characteristic, elliptical shape of the stability phase diagram that many proteins exhibit over the P/T plane. For instance, Hawley (*50*) constructed such a diagram for the reversible denaturation of chymotrypsinogen. This author proposed an equation of state for this model system, which is discussed in some detail in ref 46. Enzymes in an ongoing process of denaturation are by definition not at thermodynamic equilibrium, but several authors have nevertheless obtained similar reentrant "kinetic phase diagrams" when plotting the inactivation rate constant over the P/T plane. A good example can be found in ref 51 for butyrylcholinesterase.

Inactivation of PG under Hyperbaric Conditions. As shown above in Figure 2 and Table 1, tomato PG has demonstrated a strong resistance to heat-induced denaturation at atmospheric pressure. Figures

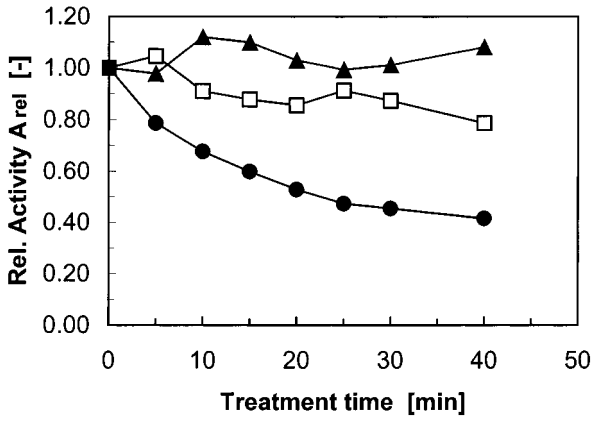

Figure 7. Inactivation of tomato PG in a juice at 45 °C and (▲) 200 MPa, (□) 300 MPa, (●) 400 MPa.

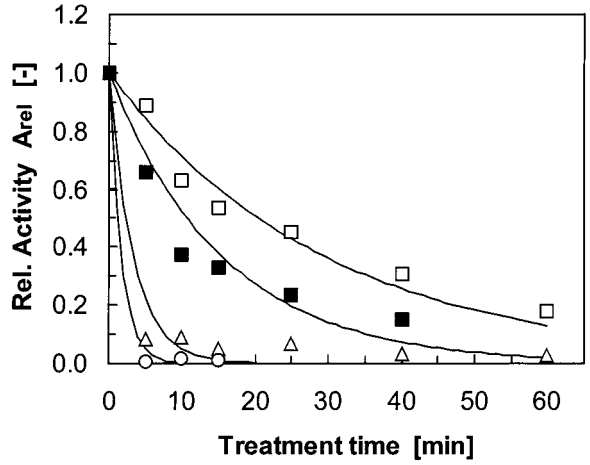

Figure 8. Inactivation of tomato PG in a juice at (□) 400 MPa/30 °C, (■) 400 MPa/50 °C, (△) 500 MPa/30 °C, (○) 600 MPa/30 °C.

7 and 8 show on the other hand that its stability is dramatically reduced as soon as pressure is raised. Contrary to PME, pressure and temperature increases both contribute to an acceleration of the inactivation rate. At the relatively "mild" pressure of 400 MPa, a 50% inactivation can be obtained in less than 30 min at 45 °C (Figure 7). Raising pressure to 500 MPa leads to a drastic enhancement of the inactivation rate and already at 30 °C, PG is inactivated below detection limit in about 20 min. Table 3 displays the values of k for PG inactivation, which correspond to the various P/T combinations that were investigated (values at 0.1 MPa are already listed in Table 1).

This type of behavior, where pressure and temperature act in synergy, can be described with a model such as the one used in ref 52 for the combined HP/T inactivation of *B. subtilis* α-amylase

$$k = A \cdot \exp\left(-\frac{B}{T}\right) \cdot \exp(C \cdot P) \qquad (8)$$

Where k is expressed in s^{-1}, T in °C and P in MPa. The following values of A, B, and C were fitted to the experimental data and found to be

$$A = 1.403 \times 10^{10}[s^{-1}]\ B = 1.055 \times 10^{4}[K]\ C = 6.049 \times 10^{3}\ [MPa^{-1}]$$

Figure 9 shows the calculated evolution of k over the

Table 3. First Order Inactivation Rate Constants k (s^{-1}) Measured for Tomato Juice PME as a Function of Pressure (MPa) and Temperature (°C)

P (MPa)	1st order inactivation rate constant k (s^{-1})				
	30 °C	40 °C	45 °C	50 °C	60 °C
100	ND[a]	ND	ND	ND	$5.26 \times 10^{-05} \pm 8.93 \times 10^{-6}$[b]
200	ND	ND	ND	ND	$8.09 \times 10^{-05} \pm 9.71 \times 10^{-6}$
300	ND	ND	$9.39 \times 10^{-05} \pm 1.17 \times 10^{-5}$	ND	$3.09 \times 10^{-4} \pm 2.81 \times 10^{-5}$
400	$1.93 \times 10^{-04} \pm 2.89 \times 10^{-5}$	$3.51 \times 10^{-04} \pm 3.86 \times 10^{-5}$	$4.43 \times 10^{-04} \pm 4.12 \times 10^{-5}$	$7.01 \times 10^{-04} \pm 4.31 \times 10^{-5}$	$2.51 \times 10^{-03} \pm 2.16 \times 10^{-4}$
500	$3.89 \times 10^{-03} \pm 5.82 \times 10^{-4}$	$4.09 \times 10^{-03} \pm 4.29 \times 10^{-4}$	ND	$3.62 \times 10^{-03} \pm 3.15 \times 10^{-4}$	$7.02 \times 10^{-03} \pm 6.39 \times 10^{-4}$
600	ND	$1.72 \times 10^{-03} \pm 2.24 \times 10^{-4}$	ND	$4.81 \times 10^{-03} \pm 3.90 \times 10^{-4}$	$7.53 \times 10^{-03} \pm 7.01 \times 10^{-4}$

[a] ND = not determined. [b] Standard errors are given.

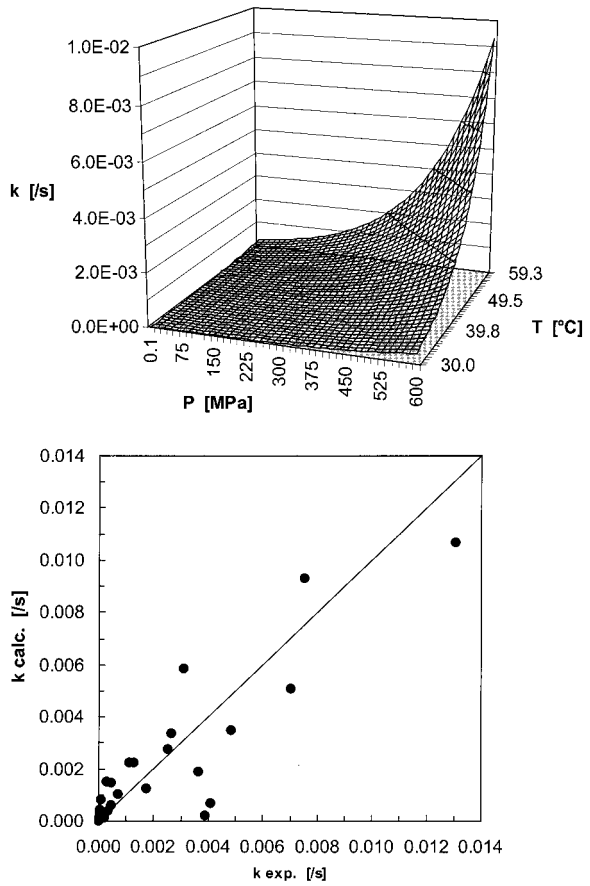

Figure 9. Evolution of the first-order inactivation rate constant k for tomato PG in a juice over the investigated P/T ranges (eq 7−8). Insert graph: Calculated vs measured values of k.

investigated portions of the P/T plane, the inserted graph displaying the quality of the fit obtained with the above model (predicted vs measured values of k). One can see that the discrepancies between calculated and measured values are larger for PG than for PME. This is due mostly to the larger uncertainty linked to PG activity measurements, but also to a poorer adequacy of the model ($r^2 = 0.7592$).

The influence of the solid content and pH of the medium on the inactivation rate of tomato PG was also investigated (Figure 10). Conditions such as 300 MPa/45 °C do not have much impact on the enzyme at the acidic physiological pH of 4.2, whether measured in the presence of tomato solids (TJ) or in a "thin" extract (TX4). On the other hand and contrary to PME, the enzyme is destabilized at higher pH values (TX7). This sensitivity could be due in part to the particular structure of tomato polygalacturonase, which is known to exist under two different forms described as PGII and PGI. PGII (45 kDa) is the heat-sensitive form of the

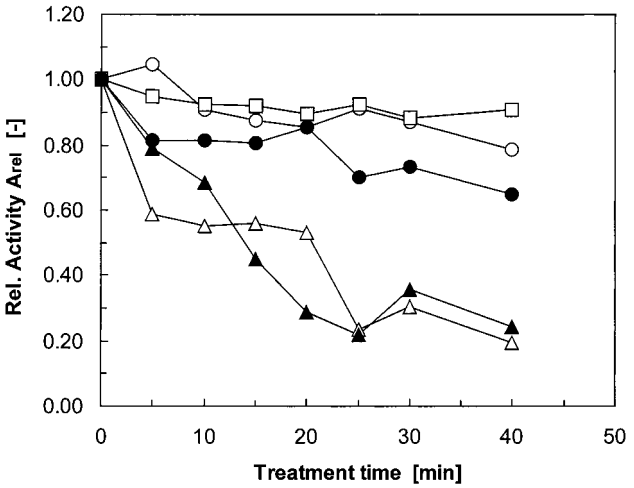

Figure 10. Residual PG activity in samples of various complexity and pH treated at (○) 300 MPa/45 °C/TJ, (□) 300MPa/45 °C/TX4, (△) 300 MPa/45 °C/TX7, (●) 400 MPa/30 °C/TX7.

enzyme, which is dramatically stabilized against thermal denaturation after combination with a 38 kDa protein known as β subunit, a process which results in the formation of the PGI species. The relative amounts of both isoforms depend strongly on the ripeness stage of the tomato fruit (*53*). Furthermore, the extraction conditions also influence the relative amounts of PGI and PGII in the resulting solution; at the physiological pH of 4.2, PGII is preferentially extracted at 0.5 M NaCl, whereas PGI prevails when sodium chloride concentration is raised to 1.25 M (*53*). At pH 11 in a 0.2 M glycine buffer, Pressey (*54*) observed that PGI was dissociated into PGII and β subunit. Raising the pH in the tomato extract has possibly altered the ratio of the different forms of the enzyme, thus resulting in a greater sensitivity to the high-pressure treatment. It is mentioned in refs 54 and 55 that PGI and PGII are basic proteins with pIs of 8.6 and 9.4 respectively (5.1 for the β-subunit). In addition to the effect of pH, it is thus likely that pressure promotes the dissociation of the β-subunit-PGII complex, a process which leads to larger amounts of PGI (the more labile enzyme) in the medium. Indeed, several studies have shown that multimeric enzymes are dissociated already under moderately hyperbaric conditions (100 MPa), this process being usually accompanied by an overall decrease in volume. A great variety of pressure impacts on enzymatic systems has been reported, but there is nevertheless a large consensus about the dissociation of many oligomeric protein systems already in the 100−250 MPa range. This hypothesis would certainly explain the overall greater sensitivity of PG to high-pressure as compared to heat.

CONCLUSION

The above examples of tomato PME and PG confirm an earlier remark that no a priori statement can be made about the pressure lability of an enzyme on the sole basis of its thermal resistance (and *vice versa*): PME is heat-labile at 0.1 MPa and dramatically stabilized under pressure, whereas the heat-resistant PG is quickly inactivated even at moderate temperatures as soon as pressure is raised. Hence, the above-presented results are somehow in contradiction with the observation made by Clark et al (*56*) that thermophilic enzymes are stabilized by pressure and heat-labile enzymes are not. These latter conclusions were however reached in a different context and for pressures not exceeding 50 MPa, a range that was not explored in this work.

The peculiar behavior of tomato PME and PG, i.e., the complete reversal of their heat-resistance under hyperbaric conditions, offers extremely interesting possibilities for the treatment of tomato-based products. With suitable combinations of pressure and temperature, it is possible to selectively inactivate PME or PG. For example, this would help avoid the addition of exogenous, purified PME to heat-treated tomato juice in order to promote texture intensification through pectin demethylation and subsequent gelification with Ca^{2+} ions.

Medium composition and salinity had little influence on the inactivation rate of PME, but this enzyme was found less sensitive to both heat and pressure when the pH was raised above its physiological value of 4.2. PG, on the other hand, became more labile at higher pH.

It is known that pressure- and heat-induced denaturation of proteins follow different patterns and thus have different impacts on protein structure, as clearly shown by Gomes et al (*57*) in the case of papain (DSC thermograms of untreated, pressurized and heated samples). Keeping in mind that the unfolding pattern can also be affected by such parameters as pH, quaternary structure, ionic strength, presence, and type of cosolutes, one ends up with a very wide and complex field of investigation. Some of these aspects are addressed by the authors of ref 58 in their review. The choice of a 1st order inactivation model is to some extent a simplification when considering the number of isoenzymes identified for tomato PME and PG and their sometimes widely diverging heat and pressure resistance characteristics. However, the model was meant to be purely descriptive and without any mechanistic implication because only overall inactivation effects could be measured in the present system. In this respect, the exponential decay seemed to fit the experimental data reasonably well.

The suggested pressure-induced dissociation of PGII could probably be evidenced by performing electrophoretic analyses under hyperbaric conditions, in a high-pressure "bomb" such as the one described in ref 22. Such an equipment was not available for this study, but would be very valuable for the investigation of the aggregation patterns that generally accompany the denaturation of proteins.

Pressure clearly adds a new dimension to the field of (food) enzyme investigation or activity control, and some of the underlying opportunities have been reviewed here. However, the impact of the treatments is at present still difficult to predict. Progress in the understanding of the stabilization mechanisms at molecular level will thus be extremely helpful in the future.

ABBREVIATIONS USED

A, enzyme activity at time t; A_{rel}, relative enzyme activity at time t [−]; A_0, enzyme activity at time zero ($A_{r0} = 1$); D_T, decimation time at temperature T [min], [s]; E_a, activation energy [kJ·mol^{-1}]; HHPT, high hydrostatic pressure treatment; HP/T, high pressure and temperature; k, first-order inactivation rate constant [s^{-1}]; k_0, preexponential factor in the Arrhenius equation [s^{-1}]; P, pressure [MPa]; PG, polygalacturonase; PME, pectin methyl esterase; s, slope of the titration curve (mL·min^{-1}); T, temperature [°C], [K]; TJ, tomato juice (pH 4.2); TX(4), clear tomato extract pH 4.2, 0.5 M NaCl; TX(7), clear tomato extract pH 7.0, 0.5 M NaCl; t, time [min], [s]; V, volume [cm^3], [L]; z, temperature increase resulting in 90% reduction of D [°C].

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LITERATURE CITED

(1) Gould, W. A. In *Tomato Production, Processing and Technology*; CTI: Baltimore, MD, 1992; pp 323−344.
(2) BeMiller, J. N. An introduction to pectins: structure and properties. In: *Chemistry and Function of Pectins*; Fishman, M. L., Jen, J. J., Eds.; American Chemical Society: Washington, 1986; pp 1−12.
(3) Chou, T. D.; Kokini, J. L. Rheological Properties and Conformation of Tomato Paste Pectins, Citrus and Apple Pectins. *J. Food Sci.* **1987**, *52*, 1658−1664.
(4) Jansen E. F.; MacDonnell, L. R. Influence of methoxyl content of pectic substances on the action of polygalacturonase. *Arch. Biochem.* **1945**, *8*, 97−112.
(5) Pressey, R.; Avants, J. K. Solubilisation of cell walls by tomato polygalacturonases: Effects of pectinesterases. *J. Food Biochem.* **1982**, *8*, 67−74.
(6) Porreta, S. New findings on tomato products. *Fruit Processing* **1996**, *2*, 58−65.
(7) McCalloch, R. J.; Nielsen, B. W.; Beavens, E. A. Factors influencing the quality of tomato paste. Pectic changes during processing. *Food Technol.* **1950**, *5*, 691−693.
(8) Smith, C. J.; Nortje, B. K. Observations on the consistency of tomato paste. *Food Technol.* **1958**, *12*, 356−358.
(9) Porreta, S.; Carpi, G.; Dall'Aglio, G.; Ghizzoni, C. Use of ultrafiltration for preparing improved tomato pulp. *Int. J. Food Sci. Technol.* **1992**, *27*, 427−433.
(10) Porretta, S.; Birzi, A.; Ghizzoni, C.; Vicini, E. Effects of ultrahigh hydrostatic pressure treatments on the quality of tomato juice. *Food Chem.* **1995**, *52*, 35−41.
(11) Shigehisa, T.; Ohmori, T.; Saito, A.; Taji, S.; Hayashi, R. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. *Int. J. Food Microbiol.* **1991**, *12* (2−3), 207−215.
(12) Arroyo, G.; Sanz, P. D.; Préstamo, G. Effect of high pressure on the reduction of microbial populations in vegetables. *J. Appl. Microbiol.* **1997**, *82* (6), 735−742.
(13) Smelt, J. P. P. M. Recent advances in the microbiology of high-pressure processing. *Trends Food Sci. Technol.* **1998**, *9*, 152−158.
(14) Ludikhuyze, L.; De Cordt, S.; Weemaes, C.; Hendrickx, M.; Tobback, P. Kinetics for heat and pressure−temperature inactivation of *Bacillus subtilis* α-amylase. *Food Biotechnol.* **1996**, *10* (2), 105−129.
(15) Knorr, D. Effects of high hydrostatic processes on food safety and quality. *Food Technol.* **1993**, *47* (6), 156−161.

(16) Mermelstein, N. L. High-pressure processing begins. *Food Technol.* **1998**, *52* (6), 104−106.
(17) Hendrickx, M.; Denys, S.; Indrawati, Ludikhuyze L.; van den Broeck, I.; Weemaes, C. The relevance of kinetic data in high-pressure processing. In *Advances in high-pressure bioscience and biotechnology (Proceedings of the international conference on high-pressure bioscience and biotechnology, Heidelberg, 30.08−03.09.98)*; Ludwig, H., Ed.; Springer Verlag: Berlin, 1999; pp 377−380.
(18) Mozhaev, V. V.; Heremans, K.; Frank, J.; Masson, P.; Balny, C. Exploiting the effects of high hydrostatic pressure in biotechnological applications. *Trends Biotechnol.* **1994**, *12*, 493−501.
(19) Cheftel, J. C. Review: High-pressure, microbial inactivation, and food preservation. *Food Sci. Technol. Int.* **1995**, *1*, 75−90.
(20) Tonello, C. Hautes pressions. Mécanismes d'actions et utilisations dans l'industrie agroalimentaire. *Rev. Gen. Froid* **1997**, N°972 (April), 41−50.
(21) Heremans, K. High-pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bio.* **1982**, *11*, 1−21.
(22) Balny, C.; Masson, P.; Travers, F. Some recent aspects of the use of high-pressure for protein investigations in solution. *High-Pressure Res.* **1989**, *2*, 1−28.
(23) Hamon, V.; Dallet, S.; Legoy, M.-D. The pressure-dependence of two β-glucosidases with respect to their thermostability. *Biochim. Biophys. Acta* **1996**, *1294*, 195−203.
(24) Arbaisah, S. M.; Asbi, B. A.; Junaisnah, A. H.; Jamilah, B. Determination of optimum conditions for pectinesterase extraction from soursop fruit *(Anona muricata)* using response surface methodology. *Food Chem.* **1996**, *55* (3), 289−292.
(25) Wood, W. A.; Kellogg, S. T. Biomass, Part B. Lignin, pectin, and chitin. *Methodol. Enzymol.* **1988**, *161*, 89−117.
(26) Pressey, R.; Hinton, D. M.; Avants, J. K. Development of polygalacturonase activity and solubilization of pectin in peaches during ripening. *J. Food Sci.* **1971**, *36*, 1070−1073.
(27) Van den Broeck, I.; Ludikhuyze, L. R.; van Loey, A. M.; Hendrickx, M. E. Effect of temperature and/or pressure on tomato pectinesterase activity. *J. Agric. Food Chem.* **2000**, *48* (2), 551−558.
(28) Athès, V.; Guerra, P.; Combes, D. Effect of soluble additives on enzyme thermo- and/or baro-deactivation. *J. Mol. Catal. B-Enzym.* **1999**, *7*, 1−9.
(29) Ogawa, H.; Fukuhisa, K.; Kubo, Y.; Fukumoto, H. Pressure inactivation of yeasts, molds, and pectinesterase in Satsuma mandarin juice: effects of juice concentration, pH, and organic acids, and comparison with heat sanitation. *Agric. Biol. Chem.* **1990**, *54* (5), 1219−1225.
(30) Tajchakavit, S.; Ramaswamy, H. S. Thermal vs microwave inactivation kinetics of pectin methylesterase in orange juice under batch mode heating conditions. *Lebensm.-Wiss. Technol.* **1997**, *30*, 85−93.
(31) Lopez, P.; Sanchez, A. C.; Vercet, A.; Burgos, J. Thermal resistance of tomato polygalacturonase and pectinmethylesterase at physiological pH. *Z. Lebensm. Unters. For. A* **1997**, *204*, 146−150.
(32) Massaguer, P. R.; Magalhães, M. A.; Tosello, R. M. Thermal inactivation of pectinesterase in papaya pulp. In *Developments in Food Engineering*, Yano, T., Matsuno, R., Nakamura, K., Eds.; Blackie Academic & Professional: London, 1994; 495−497.
(33) Laratta, B.; Fasanaro, G.; De Sio, F.; Castaldo, D.; Palmieri, A.; Giovane, A.; Servillo, L. Thermal inactivation of pectin methylesterase in tomato puree: implications on cloud stability. *Process Biochem.* **1995**, *30* (3), 251−259.
(34) De Sio, F.; Dipollina, G.; Villari, G.; Loiudice, R.; Laratta, B.; Castaldo, D. Thermal resistance of pectin methylesterase in tomato juice. *Food Chem.* **1995**, *52*, 135−138.
(35) Labib, A. A. S.; El-Ashwah, F. A.; Omran, H. T.; Askar, A. Heat-inactivation of mango pectinesterase and polygalacturonase. *Food Chem.* **1995**, *53*, 137−142.
(36) Crelier, S.; Tâche, M.-C.; Raemy, A.; Renken, A.; Raetz, E. *High pressure for the inactivation of enzymes in food products: thermal and HHPT treatment of tomato pectin methylesterase*, Poster presentation at the 1995 9th World Congress of Food Science and technology; Budapest, Hungary.
(37) Ludikhuyze, L; *High-pressure technology in food processing and preservation: a kinetic case study on the combined effect of pressure and temperature on enzymes*. PhD Dissertation N° 362. Faculty of Agricultural and Applied Biological Sciences, Catholic University of Leuven: Belgium, 1998.
(38) Miyagawa, K.; Sannoe, K.; Suzuki, K. Studies on Taka-amylase A under high-pressure treatment. II. Recovery of enzymic activity of pressure-inactivated Taka-amylase A and its enhancement by retreatment at moderate pressure. *Arch. Biochem. Biophys.* **1964**, *106*, 467−474.
(39) Defaye, A. B.; Ledward, D. A.; MacDougall, D. B.; Tester, R. F. Renaturation of metmyoglobin subjected to high isostatic pressure. *Food Chem.* **1995**, *52*, 19−22.
(40) Hernández, A.; Cano, M. P. High-pressure and temperature effects on enzyme inactivation in tomato puree. *J. Agric. Food Chem.* **1998**, *46*, 266−270.
(41) Van den Broeck, I.; Ludikhuyze, L. R.; Van Loey, A. M.; Weemaes, C. A.; Hendrickx, M. H. Thermal and combined pressure−temperature inactivation of orange pectinesterase: influence of pH and Ca^{2+}-ions. In *Advances in high-pressure bioscience and biotechnology (Proceedings of the international conference on high-pressure bioscience and biotechnology, Heidelberg, 30.08−03.09.98)*. Ludwig, H., Ed.; Springer-Verlag: Berlin, 1999; pp 377−380.
(42) Asaka, M.; Hayashi, R. Activation of polyphenoloxidase in pear fruits by high-pressure treatment. *Agric. Biol. Chem.* **1991**, *55* (9), 2439−2440.
(43) Asaka, M.; Aoyama, Y.; Nakanishi, R.; Hayashi, R. Purification of a latent form of polyphenoloxidase from La France pear fruit and its pressure activation. *Biosci. Biotech. Biochem.* **1994**, *58* (8), 1486-1489.
(44) Murao, S.; Nomura, Y.; Yoshikawa, M.; Shin, T.; Oyama, H.; Arai, M. Enhancement of activities of cellulases under high hydrostatic pressure. *Biosci. Biotech. Biochem.* **1992**, *56* (8), 1366−1367.
(45) Stapelfeldt, H.; Petersen, P. H.,; Kristiansen, K. R.; Qvist, K. B.; Skibsted, L. H. Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.* **1996**, *63*, 111−118.
(46) Michels, P. C.; Hei, D.; Clark, D. S. Pressure effects on enzyme activity and stability at high temperatures. *Adv. Prot. Chem.* **1996**, *48*, 341−376.
(47) Mozhaev, V. V.; Lange, R.; Kudryashova, E. V.; Balny, C. Application of high hydrostatic pressure for increasing activity and stability of enzymes. *Biotechnol. Bioeng.* **1996**, *52* (2), 320−331.
(48) Basak, S.; Ramaswamy, H. S. Ultrahigh-pressure treatment of orange juice: a kinetic study on inactivation of pectin methyl esterase. *Food Res. Int.* **1996**, *29* (7), 601−607.
(49) Athès, V.; Degraeve, P.; Cavaille-Lefebvre, D.; Espeillac, S.; Lemay, P.; Combes, D. Effets comparés de la pression et de la température sur la stabilité de trois β-galactosidases. *Rec. Prog. Gen. Proc.* **1997**, *58* (11), 49−54.
(50) Hawley, S. A. Reversible pressure−temperature denaturation of chymotrypsinogen. *Biochemistry* **1971**, *10*, 2436−2442.

(51) Weingand-Ziadé, A.; Renault, F.; Masson, P. Combined pressure/heat-induced inactivation of butyrylcholinesterase. *Biochim. Biophys. Acta* **1997**, *1340*, 245−252.
(52) Ludikhuyze, L. R.; van den Broeck, I.; Weemaes, C. A.; Herremans, C. H.; van Impe, J. F.; Hendrickx, M. E.; Tobback, P. P. Kinetics for isobaric−isothermal inactivation of *Bacillus subtilis* α-amylase. *Biotechnol. Prog.* **1997**, *13*, 532−538.
(53) Knegt, E.; Vermeer, E.; Bruinsma, J. Conversion of the polygalacturonase isoenzymes from ripening tomato fruits. *Physiol. Plant.* **1988**, *72*, 108−114.
(54) Pressey, R. Polygalacturonases in higher plants. In *Chemistry and function of pectins*; Fishman, M. L., Jen, J. J. Eds.; American Chemical Society: Washington, 1986; 157−174.
(55) Pressey, R. Purification and characterization of tomato polygalacturonase converter. *Eur. J. Biochem.* **1984**, *144*, 217−221.
(56) Clark, D. S.; Sun, M. M.; Giarto, L.; Michels, P. C.; Matschnier, A.; Robb, F. T. Stabilization of thermophilic enzymes by pressure. In*: High-Pressure Bioscience and Biotechnology*; Hayashi, R., Balny, C., Eds.; Elsevier Science B. V.: Amsterdam, 1996; pp 195−202.
(57) Gomes, M. R. A.; Sumner, I. G.; Ledward, D. A. Effects of high pressure on papain activity and structure. *J. Sci. Food Agr.* **1997**, *75*, 67−72.
(58) Gross, M.; Jaenicke, R. Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes (review). *Eur. J. Biochem.* **1993**, *221*, 617−630.

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