Purified Tomato Polygalacturonase Activity During Thermal and High-Pressure Treatment

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Received 30 June 2003; accepted 31 October 2003
Published online 12 February 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.10920

Abstract: Extracted tomato polygalacturonase was purified by cation-exchange chromatography (and gel filtration) and characterized for molar mass, isoelectric point, as well as optimal pH for polygalacturonase activity. The enzymatic reaction of purified tomato polygalacturonase on polygalacturonic acid as substrate was investigated during a combined high-pressure/temperature treatment in a temperature range of 25°C to 80°C and in a pressure range of 0.1 to 500 MPa at pH 4.4 (the pH of tomato-based products). The optimal temperature for initial tomato polygalacturonase activity in the presence of polygalacturonic acid at atmospheric pressure is about 55°C to 60°C. The optimal temperature for initial tomato polygalacturonase activity during processing shifted to lower values at elevated pressure as compared with atmospheric pressure, and the catalytic activity of pure tomato polygalacturonase decreased with increasing pressure, which was mostly pronounced at higher temperatures. The elution profiles of the degradation products on high-performance anion-exchange chromatography indicated that for both thermal and high-pressure treatment all oligomers were present in very small amounts in the initial stage of polygalacturonase activity. The amounts of monomer and small oligomers increased with increasing incubation times, whereas the amount of larger oligomers decreased due to further degradation. © 2004 Wiley Periodicals, Inc.

Keywords: tomatoes; polygalacturonase activity; thermal and high-pressure processing

INTRODUCTION

Enzyme-catalyzed reactions can influence the functional properties of foodstuffs in a positive or negative way. There is a relationship between changes in rheology during industrial processing of tomato-based products and modifications in the pectin structure (a cell-wall polysaccharide), caused either by chemical conversions or by the action of endogenous pectin-degrading enzymes, namely pectinmethylesterase (PME) and polygalacturonase (PG). PME catalyzes the de-esterification of pectin from which PG further catalyzes the hydrolytic cleavage of the α-1,4-hydrolytic bonds. This depolymerization leads to a drastic loss in rheology, namely a decrease in viscosity, during industrial processing and, consequently, diminishes the quality of tomato-based products (Gould, 1992). To preserve or improve the rheology of tomato-based products, in which and PME and PG are present, PG should be inactivated selectively while an initially controlled PME activity is favorable. Previous enzyme inactivation studies (i.e., in absence of substrate) have shown that, under suitable high-pressure/temperature conditions, tomato PG can be inactivated, whereas tomato PME can remain active (Crelier et al., 2001; Fachin, 2003). An additional advantage of using high pressure is the improved preservation of the nutritional and sensorial quality of processed products (Knorr, 1993).

Firming effects after preheat treatment of different fruits and vegetables at the optimal temperature for PME activity have been described in the literature and can be attributed to the activity of PME, which lowers the degree of methylation of the pectic substances. The low-methoxyl pectin formed can be cross-linked in the presence of divalent cations, such as calcium ions. In addition, the decrease in degree of methylation decreases the susceptibility of pectin to the β-elimination chain-breaking reaction during cooking (Alonso et al., 1997; Hoogzand and Doesburg, 1961; Hsu et al., 1965; Stanley et al., 1995; Steinbuch, 1976; Stolle-Smits et al., 2000; Van Buren, 1979; Van Buren and Pitifer, 1992).

Because there are no studies available on the enzymatic reaction catalyzed by tomato PG during combined high-pressure/temperature treatment, the objective of this work was to study the effect of temperature and pressure on the activity of tomato PG in the presence of the substrate polygalacturonic acid (PGA).

MATERIALS AND METHODS

Materials

Ripe tomatoes (Lycopersicon esculentum var. Flandria Prince) were chopped into small pieces, frozen in liquid
nitrogen, and stored at —80°C until use. PGA and pectin were purchased from Fluka Chemical Co. (Switzerland). All chemicals were of analytical grade.

**Extraction of Tomato PG**

The general procedure for extraction of PG involves a buffer extraction, a precipitation step with (NH₄)₂SO₄, and a dialysis step.

PG was extracted from tomatoes using a modified version of the approach used by Pressey (1986). Thawed tomatoes (~500 g) were added to cold distilled water (1:1.5 [w/v]), blended for 1 min, and homogenized for an additional minute. The homogenate was adjusted to pH 3.0 with 0.1 M HCl, stirred for 15 min, and centrifuged at 12,000 g for 20 min. The supernatant was partially purified by precipitation with (NH₄)₂SO₄. The crude extract was brought to 20% saturation, stirred for 1 h, and centrifuged at 12,000 g for 10 min. The (NH₄)₂SO₄ concentration of the supernatant was increased to 90% saturation, stirred for 1 h, and centrifuged at 18,000 g for 20 min. The resulting precipitate was dissolved in 40 mL of 0.5 M NaCl and dialyzed exhaustively against distilled water for 3 h. Finally, the enzyme solution was centrifuged at 10,000 g for 20 min and the supernatant was collected and stored at —25°C until further purification by cation-exchange chromatography and gel filtration.

**Purification of Tomato PG**

Different tomato PG isozymes, PG1 and PG2, were isolated from the crude extract using cation-exchange chromatography. In the following experiments, only PG2 was studied, as this isozyme is abundant in ripe tomatoes (Moshrefi and Luh, 1984; Pressey, 1988; Tucker et al., 1980). Moreover, the presence of PG1 in vivo remains a matter of debate (Moore and Bennett, 1994; Poisson and Brady, 1993; Pressey, 1988). For characterization (molar mass and isoelectric point) of PG2, further purification was necessary using gel filtration.

At first, 4.5 mL of crude extract was filtered (0.45-μm syringe-driven filter, Millipore, MA) before applying the sample to the top of the cation-exchange column (CIEX; Hi-Prep 16/10 SP/XL, Amersham Biosciences, Uppsala, Sweden). The column was equilibrated previously with Na-acetate buffer (40 mM, pH 4.4). Bound proteins were eluted (0.5 mL/min) with a linear salt gradient of NaCl to 1.0 M in Na-acetate buffer (40 mM, pH 4.4). Fractions with PG2 activity were pooled and concentrated by ultrafiltration (Macrosep Centrifugal Concentrator [10K], Pall Filtron Co., Northborough, MA) at 5000 g and 4°C for 75 min (J2-HS Centrifuge, Beckman, Palo Alto, CA). The concentrated PG fractions were applied to a gel-filtration column (GF; Hi Load 16/60 Superdex 75 Prep Grade, Amersham Biosciences), which was equilibrated previously with Na-acetate buffer (40 mM, pH 4.4, 0.2 M NaCl), and then eluted with the same buffer at 0.3 mL/min. Fractions with PG activity were pooled and stored at 4°C before use.

**PG Activity Assay**

The standard assay for PG activity was used to: (i) identify the fractions containing PG activity in the purification procedure; (ii) normalize the initial rate of the enzymatic reaction (Vo) at different temperature and pressure combinations; and (iii) determine the optimal pH for PG activity at atmospheric pressure.

This standard PG activity was measured spectrophotometrically based on the formation of reducing groups from PGA at 35°C and pH 4.4 (Gross, 1982). The reaction mixture in the standard assay method, consisting of 50 μL of PG solution and 350 μL of 0.3% (w/v) PGA (washed previously with 80% ethanol and dissolved in 40 mM Na-acetate buffer, pH 4.4) was incubated at 35°C for 10 min (DC30-W13, Haake, Karlsruhe, Germany). The reaction was stopped by addition of 2 mL of cold borate buffer (pH 9, 0.1 M) and 0.4 M of a 1% 2-cyanoacetamide solution to the reaction mixture, after which this mixture was incubated at 100°C for 10 min and further cooled immediately in ice water. After equilibration at room temperature, the absorbance of the solution was measured spectrophotometrically (LKB-Biocrom 4060, Pharmacia) at 276 nm and 22°C. A blank was determined in the same way by replacing the enzyme solution with Na-acetate buffer solution (40 mM, pH 4.4). To determine the optimal pH for PG activity, a blank was also taken into account for each pH value tested.

The amount of reducing groups formed was calculated using a standard curve of monogalacturonic acid (0 up to 1.4 mM), assuming that the concentration of monogalacturonic acid is proportional to the concentration of reducing groups.

**PME Activity Assay**

PME activity was measured titrimetrically at pH 7.0 and 22°C, based on the release of the amount of acid per time unit. The reaction mixture in the standard assay method consisted of 250 μL of PME sample and 30 mL of a 0.35% apple pectin solution, containing 0.117 M NaCl. During hydrolysis at 22°C, the pH was maintained at 7.0 by the addition of 0.01N NaOH using an automatic pH-stat titrator.
(Metrohm, Switzerland). The consumption of 0.01 N NaOH was recorded every 15 s during the 10-min reaction period. The activity of PME is proportional to the rate of NaOH consumption ($\Delta V_{NaOH}/\Delta t$).

**Gel Electrophoresis**

For both sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) experiments, a PhastSystem (Amersham Biosciences) was used. The molar mass of the purified tomato PG fractions was determined by SDS-PAGE under denaturing conditions using PhastGel homogeneous 20% and PhastGel Tris-tricine SDS buffer strips. The molar mass of the standards varied between 14.4 and 94 kDa. To determine the isoelectric point of the purified tomato PG by IEF, PhastGel IEF media (polyacrylamide gels) with a pH range of 3 to 9 were used. Gel staining was performed using the silver-staining protocol according to Heukeshoven and Dernick (1985).

**Enzyme Activity Studies**

**PG Activity Assay Based on Formation of Reducing Groups (Quantitative)**

The initial PG activity during thermal or combined pressure–temperature treatment was determined quantitatively by measuring the release of reducing groups, produced during the reaction of PG on PGA, as a function of treatment time. The pH during the enzymatic reaction was controlled during the reaction of PG on PGA, as a function of treatment. The pH during the enzymatic reaction was controlled at pH 4.4.

The amount of reducing groups was determined spectrophotometrically according to Gross (1982). Using this method, the enzymatic reaction was stopped by the addition of 2 mL of borate buffer (pH 9, 0.1 M) and 0.4 mL of 1% 2-cyanoacetamide to 0.4 mL of the enzyme–substrate mixture, after which this solution was incubated for a fixed period at a constant temperature (pH 4.4), was incubated for a fixed period at a constant temperature and/or pressure. The reaction was quenched by incubating the mixture at 100°C for 10 min to inactivate PG. After cooling in ice water, the mixture was diluted to 500 ppm PGA with 0.1 M NaOH, and then injected.

**Thermal Treatment**

Isothermal experiments were performed in a water bath with temperature control. The enzymatic reaction was initiated at room temperature by the addition of purified PG to a PGA solution (1:7 [v/v]). Individual small glass vials were filled with 400 μL of this PGA–PG solution and incubated in a thermostated water bath for preset time intervals. To ensure isothermal heating, an equilibration period of 1 min was necessary. After thermal treatment, the vials were withdrawn from the water bath and either the amount of reducing groups formed was determined immediately after treatment or the degradation products were analyzed. To determine initial PG activity, based on the formation of reducing groups, blanks were run for each isothermal experiment; that is, the same experiment as described earlier was performed, without the addition of PG. The temperature range studied varied from 25°C to 80°C.

**Combined Pressure–Temperature Treatment**

To perform isothermal–isobaric experiments, a laboratory pilot-scale, multivessel high-pressure apparatus (HIPU-10.000, Serial No. 95/1994, Resato, Roden, The Nether-
lands) was used. The pressure medium is a glycol–oil mixture (TR15, Resato). The apparatus allows pressurization up to 1000 MPa in combination with temperatures ranging from −20°C to 100°C. High pressure is generated using a pressure intensifier in the central pressure circuit. The temperature is controlled by a thermostated mantle, which surrounds each vessel and is connected to a cryostat. This apparatus is suited for kinetic studies because eight individual vessels (volume 8 mL, diameter 10 mm, length 100 mm) can be subjected to the same pressure and temperature levels.

The enzymatic reaction was initiated at atmospheric pressure and room temperature by the addition of purified PG to a PGA solution (1:7 [v/v]). Flexible 0.4-mL microtubes (Biozym, The Netherlands) were filled with this PGA–PG solution within 4 min and enclosed in the pressure vessels (within 4 min) that were already equilibrated at a preset temperature. Pressure was built up slowly (100 MPa/min) to minimize adiabatic heating. After reaching the desired pressure, the individual vessels were isolated and the central tubing decompressed. An equilibration period of 3 min was taken into account to allow temperature to evolve to its desired value (input value). By starting the time course of the experiment (“zero point”) after this equilibration period, the process could be considered an isobaric–isothermal treatment. At this timepoint, the vessels were decompressed after preset time intervals. After pressure release, 0.4 mL of sample was taken and either the amount of reducing groups formed was determined immediately after treatment or the degradation products were analyzed. As for the thermal treatment, for each isothermal–isobaric experiment, blanks were taken into account. The pressure range studied varied from 100 to 500 MPa in combination with temperatures from 25° to 65°C.

Data Analysis

The reaction catalyzed by PG was followed by measuring the formation of reducing groups during the thermal or the combined pressure–temperature treatment. To determine the formation of reducing groups due only to enzymatic depolymerization of PGA by PG, the amount of reducing groups formed in the blanks (PGA without addition of PG) was subtracted from the amount of reducing groups produced in the samples (PGA–PG solutions). The activity of PG (mM reducing groups per minute) was estimated from the initial linear part of the curve, which obtained by plotting the amount of reducing groups formed as a function of time and denoted \(V_0\). Because of variations in the concentration of pure PG, it is necessary to divide this activity by the amount of reducing groups formed as determined under standard assay conditions (measured spectrophotometrically at pH 4.4 and 35°C). The normalized PG activity is denoted \(V'_0\).

Once the initial rates of tomato PG-catalyzed PGA depolymerization \(V'_0\) at different temperatures are known, the temperature dependence of \(V'_0\) at a given pressure, expressed by the activation energy \(E_a\), can be estimated in the temperature area in which the reaction accelerates, using the Arrhenius equation [Eq. (1)]:

\[
V'_0 = V'_{0,ref} \exp \left( \frac{E_a}{RT} \left( \frac{1}{T_{ref}} - \frac{1}{T} \right) \right)
\]  

As a measure of the pressure dependence of \(V'_0\) at a given temperature, the activation volume \(V_a\) can be estimated using the Eyring equation [Eq. (2)]:

\[
V'_0 = V'_{0,ref} \exp \left( \frac{-V_a}{RT} \left( P - P_{ref} \right) \right)
\]  

The activation energy and the activation volume can be estimated by linear-regression analysis of the natural logarithm of \(V'_0\) versus the reciprocal of absolute temperature or versus pressure, respectively.

RESULTS AND DISCUSSION

Purification and Characterization of Tomato PG

Cation-exchange chromatography (CIEX) was used to capture PG2 from tomato crude extract. The PME and PG activity was determined in all fractions, yielding one peak of active PME fractions and two peaks of active PG fractions, denoted PG2 and PG1, respectively, based on their thermostability characteristics (Fachin, 2003) (Fig. 1). PG2 was further purified by gel filtration (GF) (Fig. 2) and characterized. The purified tomato PG2 is subsequently referred to as PG.

Tomato PG showed only one band on SDS-PAGE with a molar mass of 46.6 kDa. Using IEF, tomato PG yields an isoelectric point of >9. These data are consistent with available literature data (Ali and Brady, 1982; Moshrefi and Luh, 1984; Pogson et al., 1991; Pressey and Avants, 1973; Tucker et al., 1981).

The effect of pH on purified tomato PG activity was determined in the pH range 3.7 to 5.6 at 35°C. PG activity is maximal between pH 4.4 and 4.8 (Fig. 3), which corresponds roughly to the real pH of tomato-based products (pH 4.4). This result seems to be in agreement with available literature data (Moshrefi and Luh, 1984; Pozsár-Hajnal and Polacek-Rácz, 1975; Pressey and Avants, 1973).

Tomato PG Activity at Elevated Temperature and Atmospheric Pressure

In a preliminary test, experiments were performed with different concentrations of enzyme and substrate to identify an appropriate experimental setup. The enzyme concentration was adjusted so that the amount of reducing groups formed by PG increased linearly with time during at least 7 min (excluding dynamic phase). In this way, the activity of PG could be accurately estimated from the slope of reducing groups formed versus treatment time. For all thermal experiments (based on reducing groups formed) described
in what follows, an enzyme concentration, releasing 0.25 to 0.45 mM reducing groups when incubating PG:PGA (1:7 [v/v]) at 35°C for 10 min, and substrate concentration of 0.3% was used. Higher substrate concentrations led to (substrate) inhibition of PG.

The enzymatic reaction catalyzed by tomato PG was studied for temperatures ranging from 25°C to 80°C at pH 4.4 (40 mM Na-acetate buffer). At pH 4.4, the real pH of tomato juice, and at atmospheric pressure, the optimal temperature for activity of purified tomato PG was found to be around 55°C to 60°C (Table I). Under all temperatures tested, no chemical conversions of PGA at pH 4.4 were observed.

The temperature dependence of $V_0$ in the temperature range in which the reaction accelerated (25°C up to 50°C) could be described adequately by the Arrhenius equation, yielding an activation energy of 92.20 kJ/mol (Table I). Thus, within this temperature range, the rate of product formation accelerated with increasing temperature.

In addition to determining the initial PG activity based on the formation of reducing groups, the degradation products formed during the enzymatic reaction of tomato PG on PGA were analyzed by HPAEC. The degradation products formed during the enzymatic reaction were studied for six temperatures (30°C, 40°C, 50°C, 60°C, 65°C, and 70°C), with treatment times of 0, 5, 10, 20, and 30 min at pH 4.4.

One batch of pure tomato PG was prepared with a fixed concentration (namely 0.85 mM reducing groups formed under standard conditions), so that the same enzyme concentration was used for every treatment and, consequently, the results could be compared. At the same time, standard curves were made for monogalacturonic acid C1H2O (Fluka) and di- and trigalacturonic acid (Sigma) by plotting peak areas as a function of the concentration (mM). Within the concentration range tested, a linear relationship between peak area and concentration was observed. The slope of the standard curve of monogalacturonic acid C1H2O was significantly higher (728.7 nC/C1 min/mM) than those of digalacturonic acid (274.6 nC/min/mM) and trigalacturonic acid (258.5 nC/min/mM). The elution times for mono-, di-, and trigalacturonic acid were 7.9, 11.3, and 14.0 min, respectively.

Figure 4 is an example of an elution profile on HPAEC of the degradation products of PGA after treatment with pure tomato PG at 50°C and pH 4.4 for various incubation times (0, 5, 10, 20, and 30 min). All oligomers (DP1 to DP10) were present in very small amounts in the initial stage of the PG action, indicating that this tomato PG isozyme is an endo-acting enzyme, because an exo-acting PG cleaves only monomer units (DP1) from the nonreducing end of the substrate molecule (Di Pietro and Roncero, 1996; Dos et al., 2002; Kester et al., 1996; Tanaka et al., 2002). With increasing incubation times, the amount of monomer (DP1) and small oligomers (DP2 to DP5) increased, whereas the amount of larger oligomers (DP6 to DP10) first increased and then decreased because of further degradation. This can also be concluded from Figures 5 and 6, where, for each temperature tested, the peak areas of DP1 and DP7, respectively, are plotted as a function of the incubation time of tomato PG and PGA at atmospheric pressure and pH 4.4.
With increasing treatment times at temperatures up to 50°C inclusive, the amount of monomer (DP1) formed increased sharply, which could also be observed initially at 60°C. For a longer incubation time (>10 min) at 60°C, the rate of DP1 formation decreased due to heat inactivation of tomato PG (Fachin, 2003). At temperatures of >60°C, this heat inactivation of PG was more pronounced, resulting in a reduced amount of DP1 formed as compared with lower temperatures (Fig. 5). The larger oligomers, for example, DP7, were formed initially at all tested temperatures (Fig. 6). A steady formation of DP7 at 30°C could be observed within a 30-min reaction period. At 40°, 50°, and 60°C, a decrease in peak area of DP7 occurred after certain treatment times, indicating that more DP7 was degraded (to smaller oligomers) than (possibly) formed. Initially, at 50°C and 60°C, the rate of DP7 formation was similar, but after longer incubation times DP7 degraded further at both temperatures—faster at 50°C than at 60°C, due to heat inactivation of PG at the latter temperature. This PG inactivation was more pronounced at higher temperatures (65° and 70°C), at which DP7 was neither formed nor degraded after treatment of only 5 min.

When for all temperatures tested the peak areas of mono-, di-, and trigalacturonic acid were converted to a concentration (mM) using the corresponding standard curves, it is noted that, at all temperatures, more DP3 was formed and at a faster rate than DP2, which, in turn, was formed more and at a faster rate than DP1.

![Figure 4](image-url)  
**Figure 4.** Elution profile on HPAEC of the degradation products of PGA after treatment with pure tomato PG at 50°C and pH 4.4 for various incubation times: (1) 0 min; (2) 5 min; (3) 10 min; (4) 20 min; and (5) 30 min.

### Table I. Initial rate of the tomato PG-catalyzed PGA depolymerization ($V_0$) at pH 4.4 at different temperature and pressure combinations.

<table>
<thead>
<tr>
<th>$T$ °C</th>
<th>0.1 MPa</th>
<th>100 MPa</th>
<th>200 MPa</th>
<th>300 MPa</th>
<th>400 MPa</th>
<th>500 MPa</th>
<th>$V_0$ (cm³/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.29 ± 0.01*</td>
<td>0.30 ± 0.01</td>
<td>0.29 ± 0.03</td>
<td>0.28 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0</td>
<td>1.74 ± 0.78</td>
</tr>
<tr>
<td>30</td>
<td>0.51 ± 0.01</td>
<td>0.49 ± 0.01</td>
<td>0.38 ± 0.02</td>
<td>0.35 ± 0.02</td>
<td>0.27 ± 0.04</td>
<td>0</td>
<td>4.16 ± 0.54</td>
</tr>
<tr>
<td>35</td>
<td>1.04 ± 0.01</td>
<td>1.02 ± 0.07</td>
<td>0.89 ± 0.03</td>
<td>0.50 ± 0.01</td>
<td>0.39 ± 0.04</td>
<td>0</td>
<td>6.90 ± 1.45</td>
</tr>
<tr>
<td>40</td>
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<td>1.77 ± 0.08</td>
<td>1.37 ± 0.05</td>
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<td>0</td>
<td>5.86 ± 0.48</td>
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<td>45</td>
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<td>2.68 ± 0.09</td>
<td>1.94 ± 0.08</td>
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<td>50</td>
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<td>2.83 ± 0.22</td>
<td>2.20 ± 0.22</td>
<td>1.54 ± 0.16</td>
<td>0.58 ± 0.10</td>
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<td>12.81 ± 1.94</td>
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<td>55</td>
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<td>1.89 ± 0.16</td>
<td>0.92 ± 0.12</td>
<td>0</td>
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<tr>
<td>60</td>
<td>5.83 ± 0.53</td>
<td>2.02 ± 0.18</td>
<td>1.40 ± 0.21</td>
<td>0.39 ± 0.08</td>
<td>0</td>
<td>ND</td>
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<tr>
<td>65</td>
<td>5.10 ± 0.34</td>
<td>0.48 ± 0.07</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>$E_a$ (kJ/mol)</td>
<td>92.20 ± 3.89</td>
<td>89.67 ± 4.10</td>
<td>80.59 ± 7.56</td>
<td>76.33 ± 9.66</td>
<td>68.50 ± 13.38</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*aStandard errors of regression.

*bND, not determined.

*cProduct formation occurred linearly only up to 2 min.
of tomato PG for polymers and larger oligomers. The trimer formed is a poor substrate and was degraded only at very long incubation times. A similar conclusion was observed for PG of Aspergillus niger (Benen et al., 2003) and Fusarium moniliforme (Bonnin et al., 2003).

### Tomato PG Activity at Elevated Temperature and Pressure

The effect of elevated pressure on PG-catalyzed hydrolysis of PGA was also investigated at pH 4.4. For all pressure experiments (based on reducing groups formed) described in what follows, an enzyme concentration, releasing 0.2 to 0.4 mM reducing groups when incubating PG:PGA (1:7 [v/v]) at 35°C for 10 min, was used in the presence of a substrate concentration of 0.3%. The pressure range studied varied from 100 to 500 MPa, while temperatures varied from 25°C to 65°C.

As at atmospheric pressure, the reaction rate at elevated pressure was dependent on temperature. The optimal temperature for the PG-catalyzed hydrolysis of PGA shifted to lower values as compared with atmospheric pressure (Table I). A shift of the optimal temperature at elevated pressure has also been observed for other enzymatic reactions. For example, Van den Broeck et al. (2000) reported at elevated pressure a shift of the optimal temperature for tomato PME-catalyzed de-esterification of pectin to higher values (60°C to 65°C) as compared with atmospheric pressure (55°C).

At constant temperature, the enzymatic activity decreased with increasing pressure, this effect being more pronounced at higher temperatures. At 500 MPa, there was no tomato PG activity detected at any temperature tested. At elevated temperatures, no PG activity was observed, even at lower pressure levels. As stated in the Introduction, PG should be inactivated selectively, while an initially controlled PME activity is favorable to preserve or improve the rheology of tomato-based products. The effect of temperature and/or pressure on tomato PME-catalyzed conversion reactions at pH 4.4 has also been investigated. Comparing the results of the activity studies for both endogenous pectin-degrading tomato enzymes, PME and PG, PME activity was almost optimal and PG activity nonexistent at 55°C and 400 MPa. Yet, because at pH 4.4 the catalytic activity of tomato PME was very low (Verlent et al., data not published, extended processing times will be necessary. An exogenous, pressure-stable PME with a pH optimum for activity close to the real pH of tomato-based products (pH 4.4) is probably better for preserving or improving rheology (Christgau et al., 1996).

Under all combined temperature-pressure data tested, no chemical conversions of PGA at pH 4.4 were observed.

The temperature dependence of $V_0$ at a given pressure ($E_a$) in the temperature range in which the reaction accelerated, and the pressure dependence of $V_0$ at the different temperatures investigated ($V_0$), could be accurately described by the Arrhenius and Eyring equations, respectively (Table I). Analysis of Table I shows that activation energy decreased with increasing pressure; therefore, the temperature sensitivity of $V_0$ decreased with increasing pressure. Activation volumes for tomato PG-catalyzed PGA depolymerization were positive, indicating that pressure retards this enzymatic conversion. The activation volume increased substantially with increasing temperature; therefore, the pressure sensitivity of $V_0$ increased substantially with increasing temperature.

As for the thermal experiments, besides determination of initial PG activity, the degradation products formed during the enzymatic reaction of tomato PG (0.85 mM reducing groups formed under standard conditions) on PGA (0.3%) were investigated by HPAEC. The degradation products formed during the enzymatic reaction were studied for the same six temperatures (30°C, 40°C, 50°C, 60°C, 65°C, and 70°C) at 200 MPa, with treatment times 0, 5, 10, 20, and 30 min at pH 4.4.
Care must be taken when comparing thermal with high-pressure treatments, as the preparation times of the samples are longer (≈8 min) for high-pressure treatments, causing larger quantities of degradation products at time zero as compared with thermal treatments. Yet, the rate of formation of degradation products is higher for a thermal treatment at atmospheric pressure compared with pressure treatment at the same temperature, which is in agreement with experiments based on the determination of reducing groups formed.

In Figures 7 and 8, for each temperature tested, the peak areas of DP1 and DP7, respectively, are represented as a function of treatment time at 200 MPa and pH 4.4. The amount of monomer (DP1) formed increased with increasing treatment times for temperatures up to 50°C inclusive. At temperatures of >50°C, due to heat inactivation of tomato PG, the rate of DP1 formation decreased with increasing temperature and, for each temperature, the rate of DP1 formation diminished from a certain incubation time (Fig. 7).

The amount of DP7 increased gradually at 30°C and 200 MPa. At 40°C and 200 MPa, DP7 was formed initially by degradation of larger oligomers and polymers, but with increasing incubation times; first, DP7 was equally formed as degraded (plateau), then the amount of DP7 decreased, indicating faster degradation than formation. During the preparation time of the samples at 50°C and 60°C and 200 MPa, an extended enzymatic depolymerization of PGA occurred, so that under isobaric–isothermal conditions degradation of DP7 was observed (Fig. 8). At higher temperatures (65°C and 70°C), heat inactivation of tomato PG was more pronounced and DP7 was neither formed nor degraded during isobaric–isothermal treatment.

As for the thermal experiments, a conversion of the peak areas of mono-, di-, and trigalacturonic acid to concentration (mM) allows for the conclusion that, for all tested temperatures, at 200 MPa more DP3 is formed and at a faster rate than DP2, which, in turn, is formed more and at a faster rate than DP1.

To compare the rate of formation of degradation products (V₀) between thermal and high-pressure treatments, an example is shown for DP3 at pH 4.4 (Fig. 9). The rate of DP3 formation is higher for thermal treatment at atmospheric pressure compared with high-pressure treatment at 200 MPa, especially for temperatures of >40°C. This conclusion is in agreement with the results of the initial PG activity (V₀) studies, based on the determination of the reducing groups formed.

CONCLUSIONS

At elevated pressure, the optimum temperature for PG-catalyzed hydrolysis of polygalacturonic acid at pH 4.4 shifted to a lower value as compared with atmospheric pressure. At all temperatures tested, a lower enzyme activity (V₀) was observed at elevated pressure as compared with atmospheric pressure, and this effect of pressure was pronounced mostly at higher temperatures. We observed no initial PG activity at 500 MPa under any of the
temperatures tested furthermore, at elevated temperatures there was no PG activity even at lower pressures.

For both thermal and high-pressure treatment, with increasing incubation times, the amount of monomer and small oligomers increased, whereas the amount of larger oligomers decreased because of further degradation. Yet, for the thermal treatment, the rate of formation of degradation products was higher compared with the pressure treatment, which was in agreement with the experiments based on the determination of reducing groups formed.

Consequently, high-pressure treatment seems to be more effective than heat treatment to decrease the activity of tomato PG in the presence of this substrate to preserve or improve the rheology of tomato-based products. Preservation of the nutritional and sensorial quality of the processed products is an additional advantage when using high pressure.

NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>potential (V)</td>
</tr>
<tr>
<td>E_a</td>
<td>activation energy (J/mol)</td>
</tr>
<tr>
<td>P</td>
<td>pressure (MPa)</td>
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<tr>
<td>P_0ref</td>
<td>reference pressure (MPa)</td>
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<tr>
<td>R</td>
<td>universal gas constant (J/mol·K)</td>
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<tr>
<td>t</td>
<td>time (min)</td>
</tr>
<tr>
<td>T</td>
<td>temperature (K)</td>
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<tr>
<td>T_0ref</td>
<td>reference temperature (K)</td>
</tr>
<tr>
<td>V_0</td>
<td>initial rate of enzymatic reaction (initial PG activity) (mM reducing groups/min)</td>
</tr>
<tr>
<td>V_0ref</td>
<td>normalized V_0 at a reference temperature or pressure</td>
</tr>
</tbody>
</table>

References


