Comparative Study of the Inactivation Kinetics of Pectinmethylesterase in Tomato Juice and Purified Form

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Pectinmethylesterase (PME) extracted from tomato fruit was purified by affinity chromatography. A single peak of PME activity was observed, presenting a molar mass of 33.6 kDa, an isoelectric point higher than 9.3, and an optimal temperature and pH for activity of 55 °C and 8.0, respectively. The processing stability of purified tomato PME in buffer solution was compared to PME stability in tomato juice. In both media, thermal inactivation of PME presented first-order inactivation kinetics. PME in tomato juice being more heat-labile than purified PME. Regarding high-pressure treatment, tomato PME showed to be very pressure-resistant, revealing an outspoken antagonistic effect of temperature and pressure. To avoid cloud loss in tomato juice, a time-temperature treatment of 1 min at 76.5 °C was calculated in order to have a residual PME activity of $1 \times 10^{-4}$ U/mL.

Introduction

The quality of tomato juice depends on properties such as color, consistency, and flavor (1). Consistency refers to the viscosity of the product and the ability to hold its solid portion in suspension for the shelf life of the product, presenting almost no syneresis (2), and is related to cloud, which is defined by fine particles that remain suspended indefinitely as a result of Brownian motion. As a consequence, tomato juice cloud is responsible for some quality attributes of the juice. Pectinmethylesterase (PME), a cell wall bound enzyme present in tomato fruit, acts on pectin, resulting in cloud destabilization and loss of turbidity of the juice. PME cleaves the methyl esters of pectin, producing methanol, pectin with a low degree of esterification, and free acid. Once a degree of esterification is reached, divalent cations such as calcium can cross-link these free acid units to other free acids on adjacent pectin molecules. Such cross-linking increases the apparent molecular weight of aggregates, leading to a precipitation of the particles and a subsequent clarification of the juice (3).

Besides the fact that PME action causes cloud loss of tomato juice, it also prepares a substrate for the enzyme polygalacturonase (PG) (4), which is also present in tomato fruit. The enzyme PG catalyzes the hydrolytic cleavage of the glycosidic $\alpha$-D (1→4) bonds in the pectin molecule, leading to a decrease of viscosity of the juice due to pectin solubilization.

The inactivation of PME is currently performed by thermal treatment. Thermal processing leads to a series of known quality problems, such as changes in color, flavor, and vitamin content of the processed material. For these reasons the consumer is asking for minimally processed foods, which better preserve quality aspects of the raw materials.

High-pressure processing is a new technology that is being researched as an alternative for or complement to thermal processing, to sterilize and/or to preserve foods (5). High-pressure processing keeps the quality attributes of the raw material because covalent bonds are not changed during processing (6). It is governed by (i) the principle of Le Chatelier, which indicates that any phenomena accompanied by a volume decrease is enhanced by an increase of pressure (and vice versa), and also (ii) the isostatic rule, which states that pressure is transmitted uniformly and instantaneously throughout the sample (7).

The objective of this work was to compare PME inactivation kinetics during thermal and thermal/high-pressure processing using different degrees of food complexity, e.g. purified tomato PME and PME in tomato juice.

Materials and Methods

Raw Material. Ripened tomato fruits (Lycopersicon esculentum var. Flandria Prince) were cut, frozen in liquid nitrogen, and kept at $-80$ °C until use.

Preparation of Tomato Juice. A 500 g portion of thawed tomato was passed through a juice centrifuge, sieved (1.5 mm diameter) to remove peels and seeds, homogenized using a mechanical stirrer, and degassed in a sonicator. Tomato juice was stored in polyethylene bags of about 10 mL at −25 °C.

Extraction of Tomato PME. Thawed tomato fruit (1.0 kg) was homogenized in a blender and centrifuged at 10 000 $\times$ g for 30 min. The enzyme was extracted from the pellets using 0.2 M Tris buffer (pH 8.0) with 1 M NaCl for 2 h (1:2 w/v). The suspension was centrifuged at 10 000 $\times$ g for 1 h. The supernatant was subjected to ammonium sulfate precipitation. The fraction precipitating between 30% and 80% ammonium sulfate saturation

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was collected, dissolved in 20 mM Tris buffer, pH 7.5 (5 mL/100 g fresh material), and dialyzed against the same buffer for 24 h. After centrifugation at 18 000 \times g for 15 min, PME crude extract was stored at −25 °C. All procedures were performed at 4 °C.

**Purification of Tomato PME.** Tomato PME was purified by affinity chromatography (Akta Prime, Amersham Pharmacia Biotech, Sweden) using a PME inhibitor (PEMI), extracted from kiwi fruit, covalently immobilized on a CN-Br-Sepharose 4B matrix (Amersham Pharmacia Biotech, Sweden) \((8, 9)\). The CN-Br-Sepharose 4B resin was swollen in 1 mM HCl and washed with 0.1 M Na2CO3 buffer (pH 8.3), containing 0.5 M NaCl.

To purify the PME from kiwi fruit, commercial orange PME (Sigma) was dissolved in the same buffer and mechanically mixed with the resin for 12 h at 4 °C. After this period, the PME-CN-Br-Sepharose 4B resin was mechanically mixed with 0.1 M Tris-HCl buffer (pH 8.0) for 2 h to deactivate the unoccupied sites. Ripe kiwi fruit was peeled, homogenized in water, and centrifuged at 20 000 \times g for 20 min at 4 °C. The supernatant containing the PME was adjusted to pH 6.0 and mechanically mixed in batch with the PME-CN-Br-Sepharose 4B resin for 1 h at 4 °C. After filtration and washing with water over a glass sinter filter G3, the mixture was carefully poured in a chromatography column \((40 \text{ mm} \times 16 \text{ mm})\), and washed with 2 mM KH2PO4 buffer (pH 6.0) containing 0.5 M NaCl. The column was eluted with 20 mM Na2CO3 buffer (pH 9.5), containing 1.0 M NaCl at a flow rate of 0.125 mL/min. The fractions containing PME activity were pooled, diluted in 0.2 M NaHCO3 (1:1 v/v), pH-adjusted to 8.3, and mixed with a second CN-Br-Sepharose 4B resin for 12 h at 4 °C.

To purify tomato PME, the tomato crude extract was dissolved in 10 mM Tris buffer (pH 6.0) containing 50 mM NaCl and mixed in batch with the PME-CN-Br-Sepharose 4B resin for 1 h at 4 °C, filtered over a glass sinter filter G3, and poured carefully into a chromatography column \((40 \text{ mm} \times 26 \text{ mm})\). The column was washed with 2 mM KH2PO4 buffer (pH 6.0) containing 0.5 M NaCl and eluted with 50 mM Na2CO3 buffer, pH 9.85 containing 1 M NaCl at a flow rate of 0.125 mL/min. The fractions containing PME activity were pooled and concentrated using Centricron plus 20 (PL-10, Millipore Corporation, Bedford, MA).

**PME Activity Measurement.** The PME activity was determined by measuring the release of acid per time at pH 7.0 and 22 °C \((10)\). The reaction mixture consisted of 200 or 1000 \(\mu\text{L}\) of sample (purified and tomato juice, respectively) and 30 mL of a 0.35% apple pectin solution (70–75% esterification, Fluka, Bornem, Belgium) containing 125 mM NaCl. Before injection of enzyme solution, the pectin solution was adjusted to pH 7.0. During hydrolysis at 22 °C, the pH was maintained at 7.0 by addition of 0.01 N NaOH using an automatic pH-stat titrator (Metrohm, Switzerland). Every 15 s the consumption of 0.01 N NaOH was recorded during a 10 or 15 min reaction period. The PME activity is proportional to the rate of consumption of NaOH \((\Delta V_{NaOH}/\Delta t)\). The detection limit was reached at an activity of 0.01 mL/min. PME activity can be expressed in units (U), defined as micromoles of acid produced per minute at pH 7 and 22 °C:

\[
PME (\text{U/mL}) = \frac{V_{NaOH}}{t} \times 1000
\]

**PME Activity Measurement.** The PME activity was determined as its ability to block PME activity \((11)\). Commercial orange PME (50 \(\mu\text{L}\)) dissolved in 20 mM Tris buffer (pH 7.5) was mixed with 100 \(\mu\text{L}\) of PME solution. After incubation of the mixture for 15 min at 25 °C, the PME activity of the mixture was determined as described above. The PME activity is calculated as the difference between PME activity of a blank sample (i.e., without PME) and the residual PME activity after incubation with PME.

**Evaluation of the Purification Procedure.** The protein content was determined using bicinchoninic acid (Sigma procedure TPRO-562), forming a purple complex with an absorbance maximum at 562 nm.

The molar mass of the purified tomato PME fractions was determined by gel electrophoresis (Phastsystem Amersham Pharmacia Biotech, Sweden) under denaturing conditions in a 20% homogenous gel. The molar mass of the standards varied between 14.4 and 94 kDa.

In addition, the isoelectric point of the tomato PME fractions was determined by gel electrophoresis (Phastsystem Amersham Pharmacia Biotech, Sweden) in a pH range of 3–10.

**Thermal Treatment.** Concentrated purified tomato PME was diluted in 50 mM citrate buffer, pH 4.4, similar to the pH of tomato juice. Isothermal inactivation experiments were performed in a water bath with temperature control, in a temperature range from 62 to 80 °C. The purified PME solution was enclosed in capillary tubes (size 1.15 mm i.d. x 150 mm length, Hirschmann), while 1.2 mL of tomato juice was placed in polyethylene bags.

After preset time intervals, the samples were withdrawn from the water bath and immediately cooled in ice-water. The residual PME activities were measured within 2 h storage in ice-water.

**High-Pressure Treatment.** Isothermal/isobaric inactivation experiments were performed in a laboratory scale multivessel high-pressure equipment especially designed for kinetic experiments (HPIU-10000, Rosato, Roden, The Netherlands). The pressure medium was a glycol–oil mixture (TR15, Greenpoint oil, Resato, Roden, The Netherlands). A thermostated mantel, which surrounds each vessel, was connected to a cryostat keeping the temperature constant during the experiment. The samples (tomato juice or purified tomato PME) were placed in flexible micro tubes (0.3 mL, Elkay, Overijse, Belgium) and were enclosed in the pressure vessels already equilibrated at a certain temperature. Pressure was built up slowly (~100 MPa/min) to minimize adiabatic heating. After attaining the desired pressure, all individual vessels were isolated and the central circuit was decompressed. After a 2 min equilibration period to ensure that the preset temperature is constant \((12)\), 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 PME activity was measured within 2 h storage in ice-water.

**Data Analysis.** Enzyme inactivation often follows first-order kinetics \((13–15)\). Under isobaric–isothermal conditions, the decrease of enzyme activity as a function of treatment time can be described by:

\[
A = A_0 \exp(-kt)
\]

which can be linearized by a logarithmic transformation:

\[
\ln A = \ln A_0 - kt
\]
In food processing, it is common to express first-order reactions in terms of \( D \) and \( z \)-values \((16)\). The decimal reduction time, or \( D \)-value, is defined as the time at a given temperature and pressure, needed for a 90\% reduction of the initial activity. For first-order reactions, the \( D \)-value is inversely proportional to the inactivation rate constant:

\[
D = \frac{2.303}{k}
\]  

The decimal reduction time at a certain temperature or pressure was estimated from the slope of the regression line of \( \log(A/A_0) \) versus treatment time at constant temperature \((17)\):

\[
\log \left( \frac{A}{A_0} \right) = -\frac{t}{D}
\]  

The \( z \)-value is defined as the temperature increase necessary to obtain a 10-fold decrease of the \( D \)-value. The \( z \)-value was calculated from the negative reciprocal slope of the regression of \( \log D \) versus temperature \((17)\):

\[
\log D = \log D_{\text{ref}} - \frac{T - T_{\text{ref}}}{z}
\]  

**Results and Discussion**

**Purification of PME.** Figure 1 presents the chromatographic profile for elution of tomato PME. Purified tomato PME was eluted with a high ionic strength and high pH buffer in a single protein and activity peak.

A summary of the purification steps is reported in Table 1. Fractions with PME activity were pooled, concentrated by ultrafiltration, and analyzed using SDS-PAGE, obtaining a band with a molar mass of 33.6 kDa, and using IEF, yielding an isoelectric point higher than 9.3.

In previous studies, tomato PME was purified using a heparin-sepharose column, yielding three peaks with PME activity. These isoforms were analyzed on SDS-PAGE, showing a single protein band of 31 kDa and an isoelectric point higher than 9.3 \((18)\). Using gel filtration chromatography, molar masses of 23.8 and 24.2 kDa for the two main PME isoforms present in different varieties of cherry tomatoes were determined \((19)\). Marquis and Bucheli \((20)\) estimated the molar mass of purified tomato PME by SDS–PAGE to be 36 kDa. Our results of molar mass for purified tomato PME are in agreement with the results obtained by other authors. The difference in the number of peaks obtained during purification procedure can be explained by the fact that other tomato varieties can present different elution profiles \((18)\) and the purification method, i.e., kind of gel, chromatographic methods, and pH \((5.0–7.5)\), can contribute to these differences.

**Characterization of Purified Tomato PME.** Purified tomato PME activity was measured at temperatures between 20 and 60 °C under standard assay conditions (Figure 2). From Figure 2 it can be observed that there is an increase in PME activity to an optimum of 55 °C at pH 7.0. Figure 3 presents the influence of pH on purified tomato PME activity at 22 °C. As it can be observed, there is an increase of activity to an optimum at pH 8.0, which is in agreement with literature results \((18, 19, 21)\).

**Thermal Inactivation of Tomato PME.** Thermal inactivation of purified tomato PME and PME in tomato juice could be accurately described by a first-order model in the temperature range studied (Figures 4–6). For none of the systems was reactivation of tomato PME activity after thermal treatment observed (data not shown). Table 2 presents the kinetic parameter estimates for thermal inactivation of tomato PME in purified form and in tomato juice. In the temperature range studied, purified tomato PME in buffer solution is more thermostable compared to PME in the juice. Figure 7 depicts the temperature dependence of the decimal reduction times for thermal inactivation of pure tomato PME and PME present in tomato juice. Thermal inactivation of PME in tomato juice is less temperature-sensitive than in the purified form, as indicated by \( z \)-values of 6.2 and 4.6 °C, respectively.

In the temperature range from 55 to 80 °C, \( z \)-values for thermal inactivation of tomato PME ranging from 5 to 6.5 °C have been reported. These values are in agreement with our data as a result of the fact that purified tomato PME was dissolved in buffer at pH 4.0–4.5 \((10, 22, 23)\). However, in a higher temperature range \((70–90 °C)\), purified tomato PME was heat-treated at pH 7.5, presenting a \( z \)-value higher \((15–24 °C)\) for the

![Figure 1](image1.png)

**Figure 1.** Affinity chromatography of tomato pectinmethylesterase on PME-Sepharose column. PME elution was done with 50 mM NaCO\(_2\) buffer (pH 9.85), containing 1 M NaCl. Volume of each fraction was 4 mL, and flow rate was 0.125 mL/min. (*) UV at 280 nm, (■) PME activity.

![Figure 2](image2.png)

**Figure 2.** Effect of temperature on the activity of purified tomato PME. Assay conditions: 0.35% apple pectin (DE 75%) + 0.125 M NaCl, pH 7.

| Table 1. Purification of PME from Tomato (var. Flandria Prince) |
|-----------------|-------|-------|----------------|-------------|
| step            | total activity (U) | protein content (mg) | specific activity (U/mg) | purification fold |
| crude extract   | 16667 | 4166  | 4.0            | 1           |
| purified        | 5500  | 300   | 18.33          | 4.58        |
| purified        | 513.7 | 19.25 | 26.68          | 6.67        |
**Figure 3.** Effect of pH on the activity of purified tomato PME. Assay conditions: 0.35% apple pectin (DE 75%) + 0.125 M NaCl, 22 °C.

**Figure 4.** Thermal inactivation of purified tomato PME in 50 mM citrate buffer, pH 4.4: (o) 62 °C, (C) 64 °C, (E) 66 °C, (®) 68 °C, (x) 70 °C.

**Figure 5.** Thermal inactivation of PME in tomato juice: (x) 62 °C, (C) 64 °C, (E) 66 °C, (®) 68 °C.

**Figure 6.** Thermal inactivation of PME in tomato juice: (x) 71 °C, (C) 73 °C, (E) 75 °C, (®) 78 °C, (x) 80 °C.

**Table 2.** Kinetic Parameters for Thermal Inactivation of Purified Tomato PME and PME in Tomato Juice

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>D&lt;sub&gt;pure&lt;/sub&gt; (min)</th>
<th>D&lt;sub&gt;juice&lt;/sub&gt; (min)</th>
<th>T&lt;sub&gt;50&lt;/sub&gt; (°C)</th>
<th>k&lt;sub&gt;D&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>175.75 ± 13.70</td>
<td>49.78 ± 2.28</td>
<td>62.56</td>
<td>3.155</td>
</tr>
<tr>
<td>64</td>
<td>76.34 ± 2.65</td>
<td>16.48 ± 0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>33.83 ± 0.79</td>
<td>9.37 ± 0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>9.06 ± 0.28</td>
<td>5.28 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>3.08 ± 0.14</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>nd</td>
<td>1.20 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>nd</td>
<td>0.52 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>nd</td>
<td>0.29 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>nd</td>
<td>0.13 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>nd</td>
<td>0.062 ± 0.003</td>
<td></td>
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</tr>
</tbody>
</table>

*a Standard error. nd: not determined.

**Figure 7.** Temperature dependence of decimal reduction time for purified tomato PME (x) and PME in tomato juice (®).

Reaction but the z-values ranged between 11 and 27.8 °C, presenting a biphasic temperature dependence pattern (25). These differences may be due to maturity and the variety of tomato and also due to the difference in the assay.

Knowledge of the thermal inactivation kinetics of PME in tomato juice allows calculation of an adequate temperature–time combination to avoid enzyme-related cloud stability problems during storage. An example is given below.

Assuming that the residual PME activity should drop to $10^{-4}$ U/mL to avoid cloud loss in tomato juice (25, 26),...
assuming that the reported thermal inactivation kinetics are valid until such low residual PME activity, and assuming isothermal heat treatments, one can, for example, calculate at which temperature a process of 1 min will be adequate to reach a residual PME activity of $10^{-4}$ U/mL using eqs 5 and 6.

Assuming $t = 1$ min, $A_0 = 20$ U/mL, $A = 10^{-4}$ U/mL, we can calculate a decimal reduction time of 0.19 min. On the basis of eq 6, knowing the $x$-value of 6.24 °C, we can calculate that a $D$-value of 0.19 min corresponds to an inactivation temperature of 76.5 °C. Hence, to reduce the initial PME activity of tomato juice from 20 U/mL to $10^{-4}$ U/mL, a thermal treatment of 1 min at 76.5 °C will be sufficient.

The fact that PME in tomato juice was more heat-labile than PME in a purified form, at the same pH, can generate some discussions. First of all, the procedure to extract and purify PME or the procedure to extract the juice has an effect on enzyme activity (27). It is also known that the heat stability of an enzyme can be a function of parameters such as temperature, pressure, pH, ionic strength and nature of buffer, and concentration of enzyme or other proteins, among others. In general, an enzyme is more stable to temperature in an intact tissue or in a homogenate where its structure is protected by the presence of other colloidal material (proteins, carbohydrates, pectins, etc.) than it is in a purified form. Conditions for maximum stability can be different from crude to purified form (28).

It was observed that orange PME was more rapidly thermally inactivated in concentrated orange juice than in single-strength juice (29). On the other hand, the heat inactivation of PME in orange juice was protected when tested in concentrations of 40 and 50 °brix (30).

It seems that the protective effect of some food components related to inactivation of an enzyme is dependent on the solute concentration (for instance, brix higher than 40) as well as the source of enzyme. In our case, the tomato juice presented a brix of 5. To know which components and concentrations of the food matrix are responsible for the heat stability of tomato PME, further investigations are necessary.

**High-Pressure Inactivation of Tomato PME.** Pressure stability at 25 °C of purified tomato PME and PME in tomato juice was screened by pressurizing samples for 15 min in a pressure range from 550 to 700 MPa (Figure 8). It can be concluded that in both cases tomato PME is pressure stable in the range studied. To investigate the influence of pressure on thermal inactivation of tomato PME, a treatment of purified tomato PME at 66 °C and 0.1 MPa was compared to one at 66 °C and 600 MPa

![Figure 8](image.png)

**Figure 8.** Pressure stability at 25 °C of tomato PME after 15 min treatment time: (■) purified tomato PME, (□) PME in tomato juice.

![Figure 9](image.png)

**Figure 9.** Influence of pressure on thermal inactivation of purified tomato PME in 50 mM citrate buffer, pH 4.4: 66 °C and (e) 0.1 MPa or (0) 600 MPa.

(Figure 9). From this figure, an outspoken antagonistic effect of pressure and temperature on tomato PME inactivation can be observed. Increasing pressure from 0.1 to 600 MPa clearly retards thermal inactivation of tomato PME. A similar antagonistic effect of pressure and temperature on PME inactivation in tomato juice (62 °C/0.1 or 500 MPa) was observed (data not shown). Such antagonistic effect of pressure and temperature was already described for isothermal/isobaric inactivation of commercial tomato PME (23). Diced tomatoes using high pressure combined with elevated temperature presented significant difference in PME activity between an untreated sample and after high-pressure treatment (31). Tangwongchais et al. (32) pressurized whole cherry tomatoes up to 600 MPa for 20 min and found no significant inactivation relative to the nonpressurized control.

**Conclusion**

Tomato PME could be purified in a single-step procedure based on affinity chromatography using a PME inhibitor. Thermal inactivation of purified tomato PME and PME in tomato juice followed first-order kinetics, purified tomato PME being more heat stable than PME in tomato juice. Both purified tomato PME and PME in tomato juice were pressure-stable, with an outspoken antagonistic effect of pressure and temperature. To avoid cloud loss in tomato juice, it was postulated that an isothermal treatment of 1 min at 76.5 °C would be enough to reduce PME activity in tomato juice to the desired level. High-pressure processing offers no alternative to thermal processing for PME inactivation in tomato-based products.

**Notation**

- $A_0$: initial activity
- $A$: enzymatic activity at time $t$
- $D$: decimal reduction time (min)
- $D_{ref}$: decimal reduction time at reference temperature (min)
- IEF: isoelectric focusing
- $k$: first-order inactivation rate constant ($min^{-1}$ or $sec^{-1}$)
- $N$: normality of NaOH
- $P$: pressure (MPa)
- PG: polygalacturonase
- PME: pectinmethylesterase
PMEI: pectinmethylesterase inhibitor
SDS-PAGE: sodium dodeyl sulfate-polyacrylamide gel electrophoresis
\( t \): treatment time (min or sec)
\( t_r \): reaction time (min)
\( T \): temperature (°C)
\( T_{rel} \): reference temperature (°C)
\( U \): unit of PME
\( V \): volume of NaOH (mL)
\( V_s \): sample volume (mL)
\( z \): z-value (°C)

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References and Notes

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