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# Kinetic properties and thermal behaviour of polygalacturonase used in fruit juice clarification

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**Abstract**

Kinetic properties and thermal inactivation of polygalacturonase (PG) assayed in commercial preparations (Rapidase C80, Pectinase CCM and Pectinex 3XL) were studied. The PG activities in all samples followed Michaelis–Menten kinetics and their catalytic efficiencies were calculated. The optimum pH for enzyme activity was 4.7 for Pectinex 3XL and 4.0 for both Rapidase C80 and Pectinase CCM. The PG optimum temperature lay within the range 50–55 °C. The activation energies of Rapidase C80, Pectinase CCM and Pectinex 3XL PG were 26.5, 45.6 and 4.2 kJ mol$^{-1}$, respectively. The thermal inactivation curves were not linear in the range 40–60 °C. The biphasic curves fitted to a two-fraction first-order model suggested the presence of two groups of PG isozymes in Rapidase C80 and Pectinase CCM. Half-life values demonstrated that Pectinase PG had the largest thermal stability. Additionally, thermodynamic activation parameters, such as $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta G^{\#}$ and $\Delta S^{\#}$, were calculated.
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*Keywords:* Commercial pectinases; Inactivation kinetics; Michaelis constants; Polygalacturonase; Thermal stability; Thermodynamic activation parameters

## 1. Introduction

Plant cell degrading enzymes are used industrially to enhance the quality of products and to improve the efficiency of fruit juice and wine clarification, as well as oil extraction. Commercial enzyme preparations usually contain the main hydrolytic enzymes important for these processes, such as pectinases, cellulases and hemicellulases (Couri, Terzi, Pinto, Freitas, & Costa, 2000).

Polygalacturonase (PG, EC 3.2.1.15) is a member of the pectinase family that acts on α-1–4 linkages of polygalacturonic acid (PGA) in pectin, a cementing substance in plant cell wall, causing structural degradation (Kertesz, 1951). In most industrial applications, fungal PGs prove to be the most useful because of higher enzyme activity and optimum activity at a lower pH range, suited to most fruit and vegetable processing applications (Dziezak, 1991). In apple juice manufacturing, enzymic clarification may be carried out at 15 °C for 12 h or at 50 °C for 1 h (Lea, 1995). Thus, the higher temperature is limited by the temperature tolerance of the enzyme mixture. Many fungal PGs are thermolabile and become irreversibly inactivated at about 60 °C with a few exceptions, such as *Penicillium* (Gillespie, Cook, & Coughlan, 1990), *Rhizopus* (Ros, Saura, Saimeron, & Lencian, 1993), and *Sclerotinia* (Archer & Fielding, 1975). Optimal temperature for PG activity was in the range 30–50 °C and, for temperatures greater than 50 °C, inactivation was notable after a short period of heating (Liu & Luh, 1978; Sakai, Sakamoto, Hallaert, & Vandamme, 1993). However, PG of commercial enzymes (Pectinol A1 and Röhapect D5S, from *Röhm*) was more heat-tolerant than the purified counterpart (Ceci & Lozano, 1998).

The objective of this paper was to study the kinetic properties and thermal inactivation of polygalacturonase present in three types of different commercial pectinase preparations used for apple juice clarification. Due to irreversible thermal inactivation of enzyme (Devi & Rao,

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1996), the thermodynamic approach was not possible and a kinetic approach was therefore used to measure enzyme stability. The measurement of residual activity as a function of time and the kinetic rate constants were used to study the effect of solvent on protein stability. On this basis, an Arrhenius plot was derived and the thermodynamic parameters, such as free energy ($\Delta G^{\#}$), enthalpy ($\Delta H^{\#}$) and entropy ($\Delta S^{\#}$), were calculated.

## 2. Materials and methods

### 2.1. Materials

The enzyme preparations used without further purification were Rapidase C80 (*Gist–Brocades*), Pectinase CCM (*Biocon*) and Pectinex 3XL (*Novozyme*). In addition, polygalacturonic acid (PGA) and α-D-galacturonic acid (GA) from Sigma Chemical Co were used. Other reagents were of analytical grade (Merck).

### 2.2. Polygalacturonase assay

PG activity was determined by measuring the reducing groups liberated from orange polygalacturonic acid (Rexová-Benková, 1973). The reaction mixture contained 1 ml of 1% (w/v) polygalacturonic acid (dissolved in 0.1 M acetate buffer, pH 4.2) and 1 ml of enzyme solution. The reaction mixture was incubated at 30 °C. After 1 h of incubation, the reaction was stopped by adding 0.5 ml of 0.075 M calcium acetate at pH 4.2. The reducing sugar released was determined by the method of Somogyi (1952). The standard curve was established using α-D-galacturonic acid as reducing sugar. One unit (U) of polygalacturonase activity is defined as the amount of enzyme that releases 1 μmol of galacturonic acid per min under the assay conditions.

### 2.3. Determination of kinetic parameters

The Michaelis constants ($K_m$ and $V_{max}$ values) of polygalacturonase were determined by measuring the activity reaction rates (under the conditions given earlier) at substrate concentrations ranging from 0.01% to 0.5% (w/v). The $K_m$ and $V_{max}$ values were obtained by analysing the data according to the Hanes–Woolf equation, and catalytic efficiency was calculated as the ratio $V_{max}/K_m$ (Whitaker, 1994).

### 2.4. Effects of pH and temperature on polygalacturonase activity

The effect of pH on polygalacturonase activity was studied using three buffer solutions with pH values ranging from 2.4 to 8.0. The buffers employed in these measurements were 0.1 M phthalate (pH 2.4–4.0), 0.1 M acetic/acetate (pH 4.0–5.2) and 0.2 M tris/maleate (pH 5.2–8).

The optimum temperature for hydrolysis of polygalacturonic acid was determined by measuring the PG activity at seven different incubation temperatures over a range of 30–80 °C.

### 2.5. Thermal treatment

Samples were thermally treated at 40, 45, 50, 55 and 60 °C for up to 100 min. The residual activity was calculated as:

$$\text{Residual PG activity}, \% = 100(C_t/C_0), \quad (1)$$

where $C_t$ is the activity at time $t$ (s), and $C_0$ is the activity at time $t = 0$ s.

### 2.6. Models for PG inactivation kinetics

#### 2.6.1. First-order model

Inactivation enzymes can often be described by a first-order kinetic model, i.e., enzyme activity decreases log-linearly as a function of time as described the following equation

$$\ln(C_t/C_0) = -kt, \quad (2)$$

where $C_t$, is the enzyme activity at time $t$, $C_0$ is initial enzyme activity, $t$ is treatment time, and $k$ is the first-order inactivation rate constant.

#### 2.6.2. Multi-fraction inactivation model

Kinetic data were modelled by the multi-fraction first-order model. The implicit assumptions were that $n$ fractions of PG existed and that each fraction was inactivated independently, following first-order kinetics (Ling & Lund, 1978)

$$\text{i.e., } L_1 \to I_1, L_2 \to I_2, \ldots\ldots, L_i \to I_i, \quad (3)$$

where $L$: active enzyme, $I$: inactive enzyme. The first-order inactivation rate process is (Whitaker, 1994):

$$(\mathrm{d}C_i/\mathrm{d}t) = k_i C_i. \quad (4)$$

After integration

$$C_i = C_{0i}\mathrm{e}^{-k_i t}. \quad (5)$$

Residual activity at time $t$ is the sum of the activities of individual fractions

$$\text{i.e., } C = \sum_{i=1}^{n} C_{0i}\mathrm{e}^{-k_i t}, \quad (6)$$

where $k_i$ and $C_{0i}$ are the inactivation rate constant and activity fraction of the $i$th fraction. Eq. (6) was fitted to the thermal inactivation kinetics data for $i = 1$, 2 and 3, corresponding to first order, two-fraction first order and three-fraction first order models, using a non-linear regression routine in a Sigmaplot software package to obtain rate constants and activity fractions.

*2.7. Calculation of activation parameters*

The temperature dependence of rate constant for inactivation was analysed according to an Arrhenius plot. The activation energy ($\Delta E^{\#}$) was obtained from the slope of the Arrhenius plot (regression of logarithm of reaction rate constants vs reciprocal of absolute temperature). Activation enthalpy ($\Delta H^{\#}$) for each temperature was calculated according to

$$\Delta H^{\#} = \Delta E^{\#} - RT, \quad (7)$$

where $R$ is the universal gas constant and $T$ is the absolute temperature. The values for free energy of inactivation ($\Delta G^{\#}$) at different temperatures are calculated from the first-order rate constant of inactivation process by

$$\Delta G^{\#} = -RT \ln(kh/KT), \quad (8)$$

where $h$ ($=6.6262 \times 10^{-34}$ J s) is the Planck constant, and $K$ ($=1.3806 \times 10^{-23}$ J K$^{-1}$) is the Boltzmann constant. From Eqs. (7) and (8) the activation entropy ($\Delta S^{\#}$) for PG heat-inactivation was calculated from Eq. (9)

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T. \quad (9)$$

## 3. Results and discussion

*3.1. Effect of the enzyme and substrate concentration on PG activity*

The rate of degradation of polygalacturonic acid was linear respect to enzyme concentrations up to 10, 30 and 50 ppm in Pectinex 3XL, Rapidase C80 and Pectinase CCM, respectively (Fig. 1). Therefore the enzyme concentration of Rapidase C80 and Pectinex 3XL used in future tests were 10 and 25 ppm for Pectinase CCM.

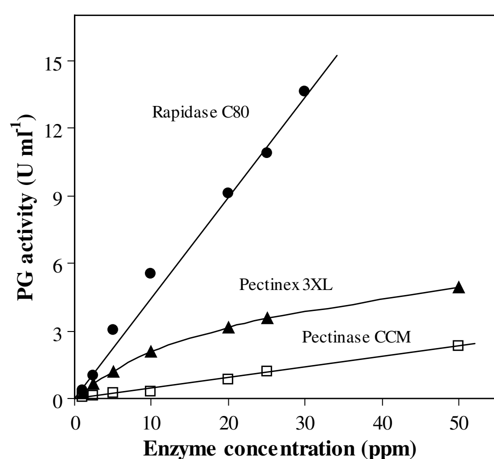


Fig. 1. Effect of pectinase concentration on polygalacturonase activity.

Table 1
Apparent Michaelis constants of polygalacturonase in Rapidase C80, Pectinase CCM and Pectinex 3XL preparations

| Enzyme | $V_{max}$[a] | $K_m$[b] | $V_{max}/K_m$[c] |
|---|---|---|---|
| Rapidase C80 | 1.91 | 0.043 | 44.4 |
| Pectinase CCM | 1.37 | 0.107 | 12.8 |
| Pectinex 3XL | 2.82 | 0.220 | 12.8 |

[a] In U.
[b] In mg ml$^{-1}$.
[c] Catalytic efficiency.

In the three enzyme preparations, polygalacturonase activity showed a typical Michaelis–Menten profile. Kinetic parameters ($K_m$ and $V_{max}$) were calculated from Hanes–Woolf plot ([$S$]/$V$ vs [$S$]) (Table 1). In all cases the graphs were linear with a correlation coefficient ($R^2$) of 0.9999–0.9865. The values of $K_m$ show that PG of Pectinase CCM and Pectinex 3XL had a relatively low affinity for its substrate compared to PG in Rapidase C80, that was much more active at low substrate concentrations. These apparent values are within the range reported for other PGs from *Lygus lineolaris* (0.060 mg ml$^{-1}$) (Agblor, Henderson, & Madrid, 1994), *Lygus hesperus* (0.032 mg ml$^{-1}$) (Agblor et al., 1994) and *Rhizopus* sp. (0.5 mg ml$^{-1}$) (Manjón, Iborra, Romero, & Canovas, 1992). However, other authors have described PGs with lower substrate affinities: from *Aspergillus niger* CH4 (2 mg ml$^{-1}$) (Acuña-Argüelles, Gutiérrez-Rojas, Viniegra-González, & Favela-Torres, 1995) and for Röhapect P (*Röhm Enzyme*) (1.8 mg ml$^{-1}$) (Pifferi, Tramontini, & Malacarne, 1989).

The catalytic efficiency value ($V_{max}/K_m$) hence provides a useful model for selecting the most efficient enzyme for an industrial process, using a fixed initial substrate concentration (Fullbrook, 1996). Examination of Table 1 indicates that the most efficient of the three commercial enzymes is the preparation Rapidase C80.

*3.2. Effect of pH and temperature on PG activity*

The effect of pH on PG activity of Rapidase C80, Pectinase CCM and Pectinex 3XL was determined by incubating the reaction mixture at pH values between 2.4 and 8 (Fig. 2). The commercial enzymes Rapidase C80 and Pectinase CCM exhibited maximal PG activity at pH 4.0, while PG in Pectinex 3XL reached a maximum at pH 4.7. The pH optimum of these enzymes is very close to reported PGs from *A. niger* (Acuña-Argüelles et al., 1995), *Lentinus edodes* (Zheng & Shetty, 2000), *Rhizopus* spp. (Elegado & Fujio, 1993) and Pectinol A1 and Röhapect D5S from *Röhm* (Ceci & Lozano, 1998).

The effect of reaction temperature on polygalacturonase activity is shown in Fig. 3. Maximal activity for Rapidase C80 was found at 55 °C whereas, for the other two commercial enzymes, the maximum was found at 50 °C. The optimal activity range was narrower in

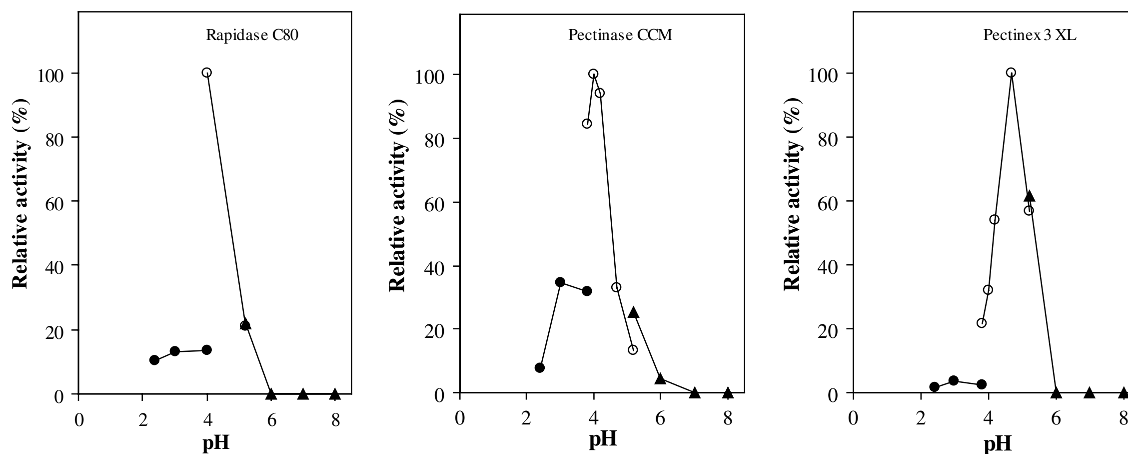


Fig. 2. Effect of pH on the polygalacturonase activity of enzyme preparations. Buffer system: (•) phthalate, (○) acetate, (▲) tris–maleate.

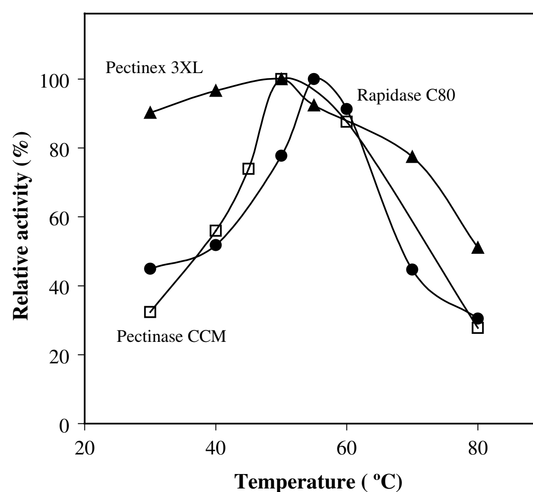


Fig. 3. Effect of temperature on polygalacturonase activity of Rapidase enzyme preparations.

Rapidase C80 and Pectinase CCM than in Pectinex 3XL. In fact, polygalacturonase activity in the range 30–70 °C in Pectinex 3XL was above of 75% of the maximal activity.

The activation energies ($E_a$) for polygalacturonase were determined using an Arrhenius model. The activation energies of PG in Rapidase C80, Pectinase CCM and Pectinex 3XL were 26.5, 45.6 and 4.20 kJ $mol^{-1}$, respectively. Similar values had been reported for polygalacturonase from *Rhizopus oryzae* CJ-2114 (8.56 kJ $mol^{-1}$) (Chung, Cho, Chum, & Choi, 1992) and *Rhizopus* spp. (27.2 kJ $mol^{-1}$) (Manjón et al., 1992).

### *3.3. Stability studies*

#### *3.3.1. General*

The use of enzymes in industrial processes may require reactions to be conducted at high temperatures in order to improve productivity and reduce microbial contamination. In this way, thermostable enzymes have been the aim of numerous studies involving the elucidation of thermal deactivation mechanisms and the development of strategies for stability enhancement (Asther & Meunier, 1990).

#### *3.3.2. Inactivation kinetics*

The kinetics of thermal inactivation of the commercial enzymes, in the temperature range 40–60 °C, were measured. The semi-logarithm graphs of residual PG activity vs heating time are shown in Fig. 4. Study of the thermal inactivation curve of commercial pectinase preparations could be relevant to industrial applications. Thus, when the process was carried out at 50 °C for 1 h, Pectinase CCM remained at 57% of the initial enzyme activity, while, Rapidase C80 and Pectinex 3XL were only maintained at 5% and 10% of the initial activities, respectively (Fig. 4).

The inactivation curves of PG in the commercial enzymes were not linear in the range of temperature studied (Fig. 4). Rather, a biphasic nature was evident, implying that inactivation of PG was not the simple first-order process commonly observed for enzyme inactivation (Richardson & Hyslop, 1985). Biphasic inactivation kinetics has been reported for different enzymes (Ortega, Busto, & Pérez-Mateos, 1998) and for PG from commercial enzymes (Ceci & Lozano, 1998) and from other sources (Lopez, de la Fuente, & Burgos, 1994).

Non-linear behaviour of inactivation curves might have been expected from a mixed enzyme preparation, where the rates of unfolding will be variable. Furthermore, biphasic thermal inactivation would result when the enzyme system consists of two groups, heat-stable and heat-labile, with differing heat stability due to enzyme aggregates formed during inactivation, each with its own thermostability or alternatively, heat-stable and heat-labile isoenzymes (Nath, 1996). Therefore, the re-

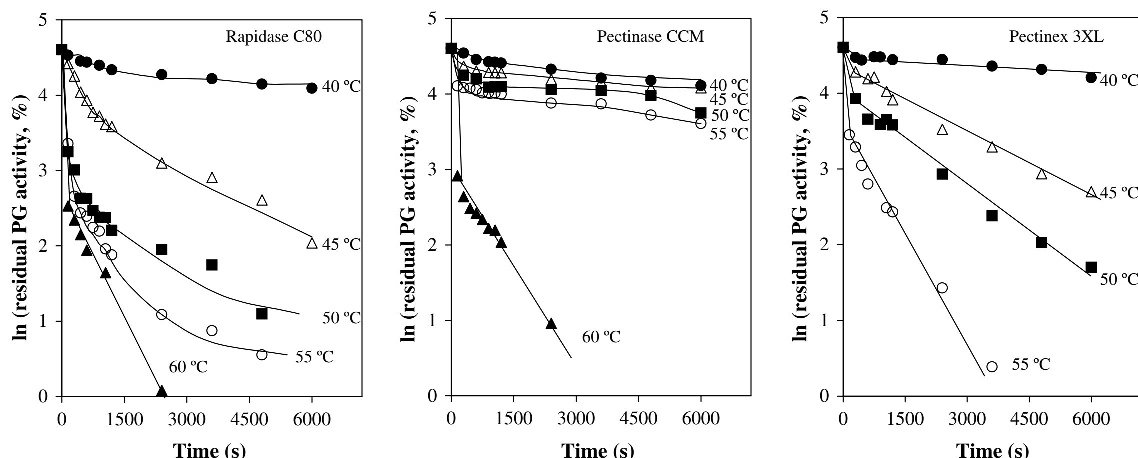


Fig. 4. Thermal inactivation kinetics of polygalacturonase activity of enzyme preparations.

Table 2
Evaluation of single fraction first order, two-fraction first order and three-fraction first order models for determination of thermal inactivation of polygalacturonase in Rapidase C80 preparation[a]

| Parameters[b] | Single fraction | Two-fraction | Three-fraction | Two-fraction with weight |
|---|---|---|---|---|
| $C_1$ | 98.3 (6.47) | 86.1 (0.96) | 46.1 ($2.56 \times 10^7$) | 85.8 (3.95) |
| $C_2$ | – | 14.0 (0.70) | 39.9 ($2.56 \times 10^7$) | 14.3 (1.02) |
| $C_3$ | – | – | 14.0 (1.03) | – |
| $k_1$ | $6.8 \times 10^{-3}$ ($1.0 \times 10^{-3}$) | $1.14 \times 10^{-2}$ ($10^{-4}$) | $1.14 \times 10^{-2}$ (4.51) | $1.16 \times 10^{-2}$ ($1.0 \times 10^{-3}$) |
| $k_2$ | – | $4.84 \times 10^{-4}$ ($4.92 \times 10^{-5}$) | $1.14 \times 10^{-2}$ (5.06) | $5.22 \times 10^{-4}$ ($4.52 \times 10^{-5}$) |
| $k_3$ | – | – | $4.83 \times 10^{-4}$ ($6.21 \times 10^{-5}$) | – |
| MSE[c] | 42.633[e] | 0.451[e] | 0.563[e] | 0.157[e] |
| $R^2$[d] | 0.9396 | 0.9995 | 0.9995 | 0.9921 |

[a] Values in parentheses are standard errors.
[b] Unit for $C_i$ is % PG activity and for $k_i$ is $s^{-1}$ where $i = 1, 2, 3$.
[c] Mean square error.
[d] Coefficient of determination.
[e] $P < 0.01$.

sults obtained in the corresponding curves could be very useful for selecting the operational conditions to achieve the optimum activity levels, depending on the industrial application.

A multi-fraction first-order model was fitted to inactivation data at each temperature. First, thermal inactivation of PG from Rapidase C80 was studied. Results of the fits are presented in Table 2 at 55 °C.

A single fraction first-order model was an inadequate fit to inactivation data because of the low coefficient of determination (0.9396) and large mean square errors (42.6) (Table 2). On the other hand, the high coefficient of determination (>0.99) and highly significant mean square errors (<0.563 at $P < 0.01$) implied that the inactivation curve was better explained by two-fraction and three-fraction first order models.

The standard errors of estimated parameters, $C_1$ ($2.56 \times 10^7$), $C_2$ ($2.56 \times 10^7$), $k_1$ (4.51) and $k_2$ (5.06), were much greater than the parameter values for the three-fraction model (Table 2). For the two-fraction first-order model, the standard errors for evaluated constants $C_1$, $C_2$, $k_1$ and $k_2$ were considerably lower at 0.96, 0.70, $10^{-4}$ and $4.92 \times 10^{-5}$, respectively. Therefore, the two-fraction model was more appropriate than the three-fraction model for the inactivation data.

Further improvement in the two-fraction model was sought by employing a weighted iterative, non-linear regression technique, the weight being the inverse of the residual squares from the first iteration (Arabshahi & Lund, 1982). Results of such analysis (Table 2) showed that the kinetic parameter values changed slightly (column two vs three), but the standard errors of the parameters decreased substantially, indicating a better fit. Therefore, the weighted iterative non-linear regression technique was applied to the inactivation data.

The evaluation revealed that a satisfactory model for inactivation of PG was a two-fraction order model, indicating the presence of two fractions of PG that differed substantially in thermo-stability. The fraction inactivated at the greater rate was designated, as phase I and the other as phase II.

On the other hand, this model was not adequate for fitting the inactivation data of PG of Rapidase C80 at 60 °C. It is possible that at this temperature, the inactivation

rate constant of the stable fraction was estimated, whereas the labile fraction was inactivated. In fact, after 150 s of treatment at 60 °C, the enzyme Rapidase C80 only retained 12% of initial activity, and for time of heating the inactivation curve was linear (Fig. 4(a)). From the slope of this curve, the inactivation constant for the phase II at 60 °C was determined.

With respect to PG inactivation of Pectinase CCM, Fig. 4(b) shows that the semi-logarithmic plots of residual activity vs time were not linear. In the temperature range 40–55 °C, the two-fraction first-order model fairly described experimental inactivation curves. This was evident from the low mean square error and high coefficients of determination (Table 3). The estimated rate constants and activity fractions are recorded in Table 3. At 60 °C, the behaviour of PG from Pectinase CCM was similar to that described for PG from Rapidase C80. The labile fraction was inactivated too quickly to accurately estimate the decline of activity and only the rate constants of stable fraction were determined.

The inactivation curves of PG in Pectinex 3XL were not able to fit a multi-fraction first-order model. However, the rate of PG activity decrease could be divided into two periods (Fig. 4(c)). The first period was characterized as a thermolabile fraction, which was inactivated too quickly to calculate the decline of activity. The second period can be defined as the thermo-resistant fraction of the enzyme, and the inactivation can be explained as a single exponential decay, indicating that inactivation followed first-order kinetics. The inactivation rate constants ($k$) were determined from the slope of such semi-log-plots.

Estimated rate constants for the inactivation of PG gave values for the enzyme half-life ($t_{1/2}$) in minutes, calculated as:

$$t_{1/2} = \ln(2)/k \ (\text{min}^{-1}). \quad (10)$$

A summary of $t_{1/2}$ values for the three enzymes is given in Table 4. It is assumed that $t_{1/2}$ values are additive (Busto et al., 1999) and therefore values from Rapidase C80 and Pectinase CCM can be calculated by combined $t_{1/2}$ values for phase I and phase II. In the range of temperature studied, the commercial enzyme more heat-stable was Pectinase CCM. In fact, this enzyme was 9.3 and 14.2 times more heat-resistant than were PG in Pectinex 3XL (at 55 °C) and Rapidase C80 (at 60 °C), respectively.

In contrast, Liu and Luh (1978) indicated that, for temperatures greater than 50 °C, inactivation was notable after a short period of heating. Devi and Rao (1998) also showed that, at 46 °C, purified polygalacturonase from *A. carbonarius* had a half-life value of 8

Table 3
Effect of increasing temperatures on thermal inactivation kinetic parameters for polygalacturonase in Pectinase CCM preparation[a]

| Temperature (°C) | Inactivation kinetic parameters[b] | | | | MSE[c] | $R^{2d}$ |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $k_1$ | $k_2$ | | |
| 40 | 18.7 (2.83) | 82.7 (5.91) | $1.40 \times 10^{-3}$ ($4.1 \times 10^{-4}$) | $2.75 \times 10^{-5}$ ($6.8 \times 10^{-6}$) | 1.544[e] | 0.9913 |
| 45 | 23.2 (1.59) | 76.6 (0.74) | $8.83 \times 10^{-3}$ ($1.62 \times 10^{-3}$) | $4.80 \times 10^{-5}$ ($3.1 \times 10^{-6}$) | 2.055[e] | 0.9876 |
| 50 | 38.8 (1.92) | 61.2 (0.78) | $1.77 \times 10^{-2}$ ($4.7 \times 10^{-3}$) | $5.21 \times 10^{-5}$ ($7.1 \times 10^{-6}$) | 3.072[e] | 0.9834 |
| 55 | 39.3 (1.76) | 60.7 (0.75) | $2.57 \times 10^{-2}$ ($1.4 \times 10^{-3}$) | $1.87 \times 10^{-4}$ ($4.9 \times 10^{-6}$) | 2.523[e] | 0.9927 |

[a] Values in parentheses are standard errors.
[b] Unit for $C$ is % PG activity and for $k_i$ is $s^{-1}$ where $i = 1, 2$.
[c] Mean square error.
[d] Coefficient of determination.
[e] $P < 0.01$.

Table 4
Thermal inactivation half-lives of polygalacturonase activity in Rapidase C80, Pectinase CCM and Pectinex 3XL preparations

| Temperature (°C) | Half life (min) | | | | |
|---|---|---|---|---|---|
| | Rapidase C80 | | Pectinase CCM | | Pectinex 3XL |
| | Phase I | Phase II | Phase I | Phase II | |
| 40 | 4.22 | 209.74 | 8.40 | 420.09 | 269.92 |
| 45 | 3.55 | 37.35 | 1.35 | 240.68 | 40.68 |
| 50 | 1.19 | 33.87 | 0.64 | 221.74 | 28.88 |
| 55 | 1.00 | 16.46 | 0.43 | 131.64 | 13.45 |
| 60 | – | 7.80 | – | 61.78 | – |

min. Therefore, the commercial enzymes assayed in this work were more heat-tolerant than purified fractions. This phenomenon was attributable to the thermo-protective action of impurities.

### 3.3.3. Temperature-dependence of commercial PG inactivation

There is surprisingly little information published concerning transition state parameters for PG heat-inactivation. Estimates of $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ for PG from *Lygus* spp., *A. carbonaius* and *A. niger*, were reported by Agblor et al. (1994), Devi and Rao (1998) and Naidu and Panda (2003), respectively. Fig. 5 shows Arrhenius plots for PG heat-inactivation at 40–60 °C. The entire graphs were linear. The activation parameters, such as $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta G^{\#}$ and $\Delta S^{\#}$, are given in Table 5.

All $\Delta G^{\#}$ values in Table 5 are almost identical and are of the order of magnitude expected for protein denaturation (Busto et al., 1999). The large activation enthalpy values are also characteristic of protein denaturation reaction (Brown & Yada, 1991; Owusu & Berthalon, 1993; Stearn, 1949). Therefore, enzyme unfolding may be the rate-determining step for the irreversible thermoinactivation of PG under the conditions assayed.

At moderate temperatures, the rate-limiting step for the irreversible heat-inactivation of enzymes is the formation of an unfolded enzyme (U) state, describing irreversible inactivation as a two-stage reaction (Ahern & Klibanov, 1988; Owusu & Berthalon, 1993; Busto et al., 1999):

$$\mathbf{N} \Longleftrightarrow \mathbf{U} \rightarrow \mathbf{I} \text{ (and } \mathbf{N} \rightarrow \mathbf{I} \text{ overall)}, \quad (11)$$

where **N** is the native conformation for PG, **U** is the heat-unfolded enzyme and **I** is the irreversible inactivated PG. Results in Table 5 show thermodynamic parameters associated with the formation of a transition state (**Tn***) according to Eq. (12):

$$\mathbf{N} \leftrightarrow \mathbf{Tn}^{*} \leftrightarrow \mathbf{U}. \quad (12)$$

Thus, $\Delta H^{\#}$ and $\Delta S^{\#}$ are, respectively, the heat and entropy change for the **N** → **Tn*** reaction. The two parameters provide a measure of the number of non-covalent bonds broken and the net enzyme/solvent disorder change associated with the **N** → **Tn*** transition, respectively. The number of non-covalent bonds broken to form the **Tn*** state is difficult to assess. There is some uncertainty connected with the bond energies and relative importance of various non-covalent bonds in the **N** state. Assuming a hydrogen or hydrophobic bond, removal of a $–CH_2$ group from solvent contact, strength of about 5.4 kJ mol$^{-1}$ (Pace, 1992; Shirley, Stanssens,

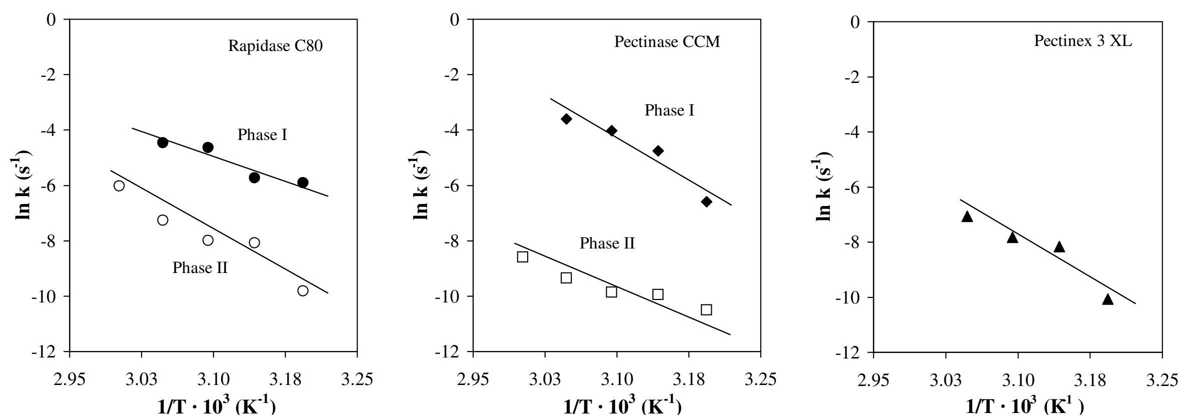


Fig. 5. Arrhenius plot for heat-inactivation of polygalacturonase of enzyme preparations.

Table 5
Thermodynamic parameter values of thermal inactivation of polygalacturonase in Rapidase C80, Pectinase CCM and Pectinex 3XL preparations at 40–60 °C

| Enzyme | $\Delta E^{\#}$ (kJ mol$^{-1}$) | $\Delta H^{\#}$ (kJ mol$^{-1}$) | $\Delta G^{\#}$ (kJ mol$^{-1}$) | $\Delta S^{\#}$ (J mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|
| *Rapidase C80* | | | | |
| Phase I | 92.5 | 89.9 | 92.1–92.7 | –7.86 |
| Phase II | 145 | 143 | 102–98.5 | 130 |
| *Pectinase CCM* | | | | |
| Phase I | 166 | 163 | 93.9–90.4 | 222 |
| Phase II | 76.6 | 73.9 | 104–105 | –95.5 |
| *Pectinex 3XL* | 160 | 157 | 103–99.8 | 176 |

Hahn, & Pace, 1992), the formation of the **Tn*** state in PG is accompanied by the disruption of up 30 non-covalent bonds.

Positive $\Delta S^{\#}$ values also suggest that enzyme unfolding is the rate determining step for the irreversible thermoinactivation of native PG of Rapidase C80 (II fraction), Pectinase CCM (I fraction) and Pectinex 3XL (Table 5). There is an increase in entropy during the conversion of the **N** state to the **Tn*** state. However, in the inactivation of PG of Rapidase C80 (I fraction) and Pectinase CCM (II fraction), the negative $\Delta S^{\#}$ values may be accounted for by possible aggregation of the partially unfolded enzyme molecules (Owusu, Makhzoum, & Knapp, 1992), which predominate during the exposure of protein to high temperatures (Dannenberg & Kessler, 1988).

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