J. Microbiol. Biotechnol. (2010), **20**(7), 1077–1085
doi: 10.4014/jmb.0908.08008
First published online 19 May 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Biochemical and Thermal Stabilization Parameters of Polygalacturonase from *Erwinia carotovora* subsp. *carotovora* BR1

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Received: August 9, 2009 / Revised: March 1, 2010 / Accepted: March 2, 2010

With an emphasis on its thermal behavior with different pHs and salts, the kinetic and thermodynamic parameters of the purified polygalacturonase (PG) from *E. carotovora* subsp. *carotovora (Ecc)* BR1 were studied, as the characterization of an enzyme is significant in the context of burgeoning biotechnological applications. The thermodynamic parameters for polygalacturonic acid hydrolysis by the purified PG were ΔH^*=7.98 kJ/mol, ΔG^*=68.86 kJ/mol, ΔS^*=−194.48 J/mol/K, ΔG_{E-S}=−1.04 kJ/mol, and ΔG_{E-T}= −8.96 kJ/mol. In addition, its turnover number (k_{cat}) was 21/sec. The purified PG was stable within a temperature range of 20–50°C and was deactivated at 60°C and 70°C. The thermodynamic parameters (ΔH^*, ΔG^*, ΔS^*) for the irreversible inactivation of the PG at different temperatures (30–60°C) were determined, where the effectiveness of various salts and different pHs (4–8) for the thermal stability of the PG were also characterized. The efficacy of various salts for the thermal stability of the PG was in the following order: $MgCl_2$>$BaCl_2$>KCl>$CaCl_2$>NaCl. Therefore, the present work presents the biochemical, substrate hydrolysis thermodynamics and the thermal stabilization parameters of the PG from *Ecc.*

Keywords: Polygalacturonase, *Erwinia carotovora*, thermodynamics, kinetic, thermal stabilization

Pectinases have tremendous potential in the enzyme industry, especially in relation to the food, textile, waste treatment, and paper industries [16]. They also aid in maintaining an ecological balance by causing the decomposition and recycling of waste plant materials [13].

Microbial pectinases account for almost 25% of global food enzyme sales, where nearly all the commercial preparations of pectinases are produced from fungal sources, and mostly from *Aspergillus niger* [13]. Thus, with the extended application of pectinases in various fields, there is an increasing demand for the discovery of new strains producing pectinases with novel properties. Therefore, determining the characteristics of pectinase is essential for its efficient and effective application [33]. Biochemical and thermal stabilization characterizations can help to establish additional information required to maintain the desired level of enzyme activity over a long period of time. Moreover, these are important parameters taken into account in the selection and design of an enzyme [11].

Plant pathogenicity and the spoilage of fruit and vegetables by rotting are some of the manifestations of pectinolytic enzymes [13]. For example, the extracellular pectinolytic enzymes produced by *E. carotovora* subsp. *carotovora* (*Ecc*) and other soft-rot *Erwinia* sp. are required for the induction of tissue-macerating (soft-rotting) disease in a wide variety of plants [1, 29]. Moreover, pectin-degrading enzymes, particularly endopolygalacturonases (endo-), are among the initial glycanases synthesized and secreted during the majority of phytopathogenic microbial infections [10]. There is also strong correlative evidence supporting the involvement of endo-PG in causing soft-rotting or tissue maceration, making it a possible virulence determinant of soft-rot *Erwinia*. PG (E.C. 3.2.1.15) is a member of the pectinase family that acts on the α-(1,4)-linkages of polygalacturonic acid (PGA) in the pectin present in plant cell walls, causing structural degradation [30].

Although the bacterial pectinases produced by *Bacillus* spp. have already been the focus of many publications for varied applications [13], the present study examined the potential of the PG produced by *Ecc* BR1 for industrial application. Accordingly, this report is the first kinetic and thermodynamic characterization of the PG produced by *Ecc* BR1 and of its thermal behavior with different pHs and salt conditions.

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Materials and Methods

Microorganism and Culture Conditions

The bacterial strain *E. carotovora* subsp. *carotovora* (*Ecc*) BR1 used in this study was isolated from macerated tissue of Brinjal fruit (*Solanum melongena* var. *esculentum*), and identified on the basis of 16S rRNA gene sequencing. The gene sequence has been deposited in the NCBI GeneBank (Accession No. FJ187821). The bacterium was cultivated and routinely maintained on a nutrient agar medium containing 0.5% pectin.

Production and Purification of PG

A liquid medium containing (g/l) the peptic digest of animal tissue (5.0), sodium chloride (5.0), beef extract (1.5), yeast extract (1.5), and pectin (5.0) with the pH adjusted to 6.5 was sterilized by autoclaving at 121°C, 15 lbs for 15 min. A 1% inoculum of *E*cc BR1 (1×10^8 CFU/ml) was then added to 500 ml of the medium in a 2-l Erlenmeyer flask and incubated for 24 h at 30°C on a rotary shaker maintained at 180 rpm. The culture was harvested at the end of the growth phase, centrifuged at 15,880 ×*g* for 15 min at 4°C, and the supernatant precipitated with acetone [1:1 (v/v)]. After storing for 4 h at 4°C, the precipitate was centrifuged, dissolved in a 50 mM sodium acetate buffer (pH 5.0), dialyzed against a 5 mM sodium acetate buffer (pH 5.0), and finally chromatographed on a CM-cellulose column (1.5 cm×12 cm) at a flow rate of 0.5 ml/min using a linear gradient elution technique with the same buffer while increasing the ionic strength from 5 to 500 mM [24].

SDS–PAGE, Isoelectric Focusing (IEF), and Activity Staining Assay

The SDS–PAGE was performed using a 12% polyacrylamide gel according to the method of Laemmli [17]. Protein molecular mass markers (Banglore Genie, India; PMWH range: 29–205 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 43 kDa; bovine serum albumin, 66 kDa; myosin-rabbit muscule, 205 kDa) were run alongside the samples. After the electrophoresis, the gel was stained using silver salts [28]. The IEF was performed as described by Palomares and Préstamo [26] using a Protean IEF system (BioRad), with 7-cm IPG strips (ReadyStrip IPG Strip, BioRad) within a pH range of 3–10, and IEF standards (pI range 4.45–9.6; BioRad) as the protein pI markers. The activity staining assay of the polygalacturonase was performed using polygalacturonic acid as the substrate incorporated in a 12% polyacrylamide gel. Following electrophoresis, the gel was incubated overnight at 30°C in a 0.05 M sodium acetate buffer (pH 5.0) contained in 0.2 M NaCl and then stained with 0.01% (w/v) Toluidine blue-O [20].

Polygalacturonase Assay

The polygalacturonase activity was determined by measuring the reducing sugar released as a result of the hydrolysis of polygalacturonic acid using a dinitrosalicylic acid (DNS) reagent [21]. A reaction mixture containing 200 µl of 0.5% (w/v) PGA in a 50 mM sodium acetate buffer (pH 5.0), and 100 µl of the appropriately diluted enzyme solution was incubated at 40°C for 20 min and the absorbance of the reaction products monitored at 540 nm. The reducing sugars formed were quantified using D-galacturonic acid as the standard. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of D-galacturonic acid per minute at 40°C, pH 5.0.

Effects of pH and Temperature on Activity

The effect of the pH on the PG was determined by measuring the activity at 40°C for 20 min when using different buffers (50 mM), namely, a phthalate buffer (pH 2.6–3.0), Na-acetate buffer (pH 4.0, 5.0, 5.4), phosphate buffer (pH 6.0,7.0), and glycine-NaOH buffer (pH 8.0, 9.0, 10.0). The optimum temperature and activation energy (E_a) were determined by incubating an appropriate amount of the enzyme with 0.5% PGA at various temperatures ranging from 20° to 70°C in a 50 mM Na-acetate buffer (pH 5.0) for 20 min. The E_a was calculated using an Arrhenius plot [31], where the E_a was calculated from the slope of a linear plot of 1000/T versus ln[PG activity], E_a=–slope×R, where R (gas constant)=8.314 J/K/mol. The effect of the temperature on the rate of the reaction was expressed in terms of the temperature quotient (Q_{10}), which is the factor by which the rate increases because of a rise in temperature of 10°C. Q_{10} was calculated by rearranging equation [22]:

$$Q_{10} = \text{antilog}_e\left(\frac{E\times 10}{RT^2}\right) \quad (1)$$

where E=E_a=activation energy.

Estimation of Kinetic Parameters

The Michaelis constants (K_m and V_{max} values) were determined by measuring the activity reaction rates (under the above assay conditions) at substrate concentrations ranging from 0.01 to 0.5% (w/v) PGA with a fixed amount of the enzyme in a 50 mM Na-acetate buffer (pH 5.0) at 40°C for 20 min. The K_m and V_{max} values were obtained from a Lineweaver–Burk plot (1/[S] versus 1/V), allowing the catalytic efficiency (*i.e.*, the ratio V_{max}/K_m), and kinetic constants (k_{cat}, k_{cat}/K_m) to be determined [22]. Meanwhile, the substrate specificity was established using a 0.5% (w/v) solution of pectic substrates (*viz.*, PGA and pectin) with a 28%, 35%, and 95% degree of esterification (DE). The specificity in terms of the specific activity was measured using a PG assay.

Estimation of Thermodynamic Parameters for Polygalacturonic Acid Hydrolysis

The thermodynamic parameters for the substrate hydrolysis were calculated by rearranging Eyring's absolute rate equation derived from the transition state theory [4, 22]:

$$k_{cat} = \left(\frac{k_b T}{h}\right) e^{(-\Delta H^*/RT)} e^{(\Delta S^*/R)} \quad (2)$$

where k_b is Boltzmann's constant (R/N)=1.38×10^{-23} J/K, R is the gas constant=8.314 J/K/mol, N is Avogadro's number=6.02×10^{23}/mol, T is the absolute temperature (K), h is Planck's constant=6.626×10^{-34} J s, ΔH^* is the enthalpy of activation, and ΔS^* is the entropy of activation:

$$\Delta H^* = E_a - RT \quad (3)$$

$$\Delta G^* \text{ (free energy of activation)} = -RT \ln\left(\frac{k_{cat} h}{kbT}\right) \quad (4)$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \quad (5)$$

where the E_a of the enzyme for the substrate hydrolysis was determined using Arrhenius' model, as described above.

The free energies for the substrate binding and transition state formation were calculated using the following derivations:

$$\Delta G^*_{E-S} \text{ (free energy of substrate binding)} = -RT \ln K_a \quad (6)$$

where $K_a = 1/K_m$:

$$\Delta G^*_{E-T} \text{ (free energy for transition state formation)} = -RT \ln \left(\frac{k_{cat}}{K_m}\right) \quad (7)$$

Thermal Stability Assay

The thermal inactivation was determined by incubating the enzyme at 20°, 30°, 40°, 50°, 60°, and 70°C. Aliquots were withdrawn at 10-min intervals, cooled on ice for 1 h, and assayed for PG activity.

Effects of pH or Salts on Thermal Stability

A pH range from 4.0 to 8.0 was selected for determining the thermal stability. The pH of the enzyme solutions was adjusted with acetic acid (1 M) or NaOH (1 M), followed by a thermal stability assay. The selected salt concentrations were 0.1 mM $CaCl_2$, 0.1 M NaCl, 5 mM $MgCl_2$, 0.15 M KCl, and 0.5 mM $BaCl_2$ on the basis of earlier observations by Nasuno and Starr [24]. The stability of the purified enzyme was studied by incubating the enzyme with each salt separately at 4°C for 10 min. The remaining unbound salt was removed by dialysis for 1 h, followed by a thermal stability assay.

Estimation of Deactivation Rate Constant

The residual activity was determined by comparing the enzyme activity after heating with that of the freshly prepared unheated enzyme. The deactivation rate of the enzyme was calculated using a first-order expression [23]:

$$\frac{dE}{dt} = -K_d E \quad (8)$$

So that

$$\ln\left[\frac{E_t}{E_0}\right] = -K_d t \quad (9)$$

The K_d (deactivation rate constant or first-order rate constant) values were calculated from a plot of time (t) versus $\ln[E_t/E_0]$ at a particular temperature.

The half-life of the enzyme was defined as the time required for the enzyme to lose half of its initial activity, as given by

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (10)$$

Estimation of Thermodynamic Parameters for Thermal Deactivation

The thermodynamic parameters were calculated by rearranging the Eyring absolute rate equation [15]. The ΔH^* and ΔS^* values were calculated from the slope and intercept of a 1/T versus $\ln[K_d/T]$ plot, respectively. Therefore

$$\Delta H^* = -(\text{slope})R \quad (11)$$

$$\Delta S^* = R\left[\text{intercept} - \ln\left(\frac{K_b}{h}\right)\right] \quad (12)$$

The free energy change was calculated using the following relationship:

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (13)$$

The energy of deactivation was estimated using Arrhenius' equation:

$$K_d = A e^{(-E/RT)} \quad (14)$$

Therefore

$$\ln[K_d] = -\frac{E}{RT} + \ln A \quad (15)$$

The energy (E) involved in the deactivation process was calculated from the slope of a linear plot of 1/T versus $\ln[K_d]$ [7], $E = -\text{slope} \times R$, where R (gas constant)=8.314 J/K/mol.

The thermal stability of the enzyme in the presence of different salts and at various pHs was determined by heating the enzyme in the presence of the salt or at a specific pH in sealed tubes for different times at 30°, 40°, 50°, and 60°C. The enzyme activity was measured before and after the incubation period at each temperature to demonstrate the residual activity under the respective conditions. All the experiments were conducted in triplicate and the results shown are the mean values.

RESULTS AND DISCUSSION

Purification and Characterization of PG

The purification of the PG produced from a locally (Gujarat, India) isolated *Ecc* BR1 strain grown under submerged conditions is summarized in Table 1. The purification to apparent homogeneity was completed in two steps, checked using SDS–PAGE stained with silver salts and activity staining, and the purified PG was found to have a pI value of 7.0 with a molecular mass of 43 kDa (Fig. 1).This pI value differed from those reported previously for other isoforms of the PG from an *Ecc* EC1 strain [14]. An overall purification of up to 20.6-fold with a 6.1% recovery was achieved (Table 1).

The optimum PG activity was observed within a range of pH 4.0–6.0, with the maximum at pH 5.0 and a

Table 1. Purification of *Ecc* BR1 polygalacturonase.

Purification steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	% Yield
Crude enzyme	200	1,237	0.16	1	100
Acetone precipitation	168	76.8	2.2	13.7	84
CMC column chromatography	12.14	3.7	3.3	20.6	6.1

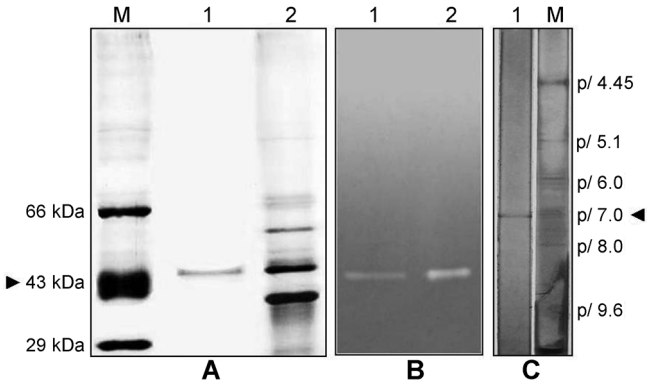

Fig. 1. SDS–PAGE analysis (**A**), activity staining zymogram (**B**), and IEF analysis (**C**) of PG from *Ecc* BR1 during purification steps.
Lane, M, Protein M_W/p*I* markers; 1, Purified PG; 2, Dialyzed acetone precipitates.

temperature of 40°C (Fig. 2A and 2B). The E_a for the substrate hydrolysis of the purified PG was 10.6 kJ/mol, which was lower than the values previously reported for PGs from other sources [18]. The overall Q_{10} (temperature quotient) of the purified PG was 1.03 between 20°C and 70°C.

Determination of Kinetic Parameters of Purified PG

The purified PG showed a typical Michaelis–Mentan profile, and the K_m and V_{max} values for the PG determined using a Lineweaver–Burk plot for the hydrolysis of polygalacturonic acid at 40°C were 0.67 mg/ml and 2.38 μM/ml/min, respectively. The apparent K_m value was in proximity to previously reported values for other PGs from *Rhizopus* sp. (0.5 mg/ml) [18], *Burkholderia cepacia* (0.49 mg/ml) [19], and *Sclerotinia sclerotiorum* (0.8 mg/ml) [6]. However, the estimated K_m value was 7.0 and 1.2 times lower than that previously determined for the polygalacturonase from *Saccharomyces cerevisiae* and *S. sclerotiorum*, indicating that the current polygalacturonase had a higher affinity for PGA than the polygalacturonases from the other two microorganisms [13]. The turnover number k_{cat} was 21/sec, and the apparent second-order rate constant (k_{cat}/K_m) was 31.34 (Table 2). The PG from *E*cc BR1 exhibited a specific activity of 6.84 U/mg, determined from the kinetic parameters. The catalytic efficiency value (V_{max}/K_m) of the purified PG from *E*cc BR1 was 3.55 μmol/min/mg substrate (Table 2), which is useful for measuring the efficiency of the enzyme in commercial applications [25]. The purified PG was capable of hydrolyzing a variety of different pectic substrates with different DE percentages, showing a high substrate specificity towards PGA (3.1 U/mg protein) and 35% DE pectin (3.01 U/mg protein), and a lower substrate specificity towards 28% DE pectin (1.0 U/mg protein) and 95% DE pectin (0.4 U/mg protein). Thus, there was a concomitant decrease in the PG activity with an increase in the DE percentage in the pectic substrate.

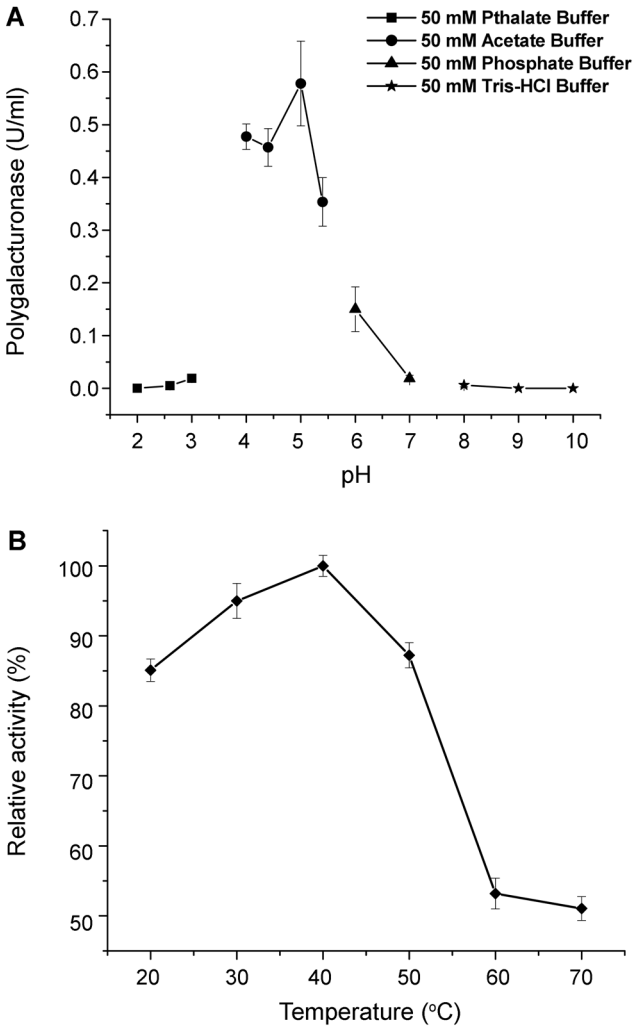

Fig. 2. Effects of pH (**A**) and temperature (**B**) on the activity of purified PG from *Ecc* BR1.

Determination of Thermodynamic Parameters of Purified PG for Polygalacturonic Acid Hydrolysis

There is relatively little information on the thermodynamic parameters for the substrate hydrolysis of PG. Thus, the enthalpy of activation (ΔH^*), Gibbs free energy (ΔG^*), and entropy of activation (ΔS^*) for polygalacturonic acid hydrolysis by the purified PG from *Ecc* BR1 were calculated (Table 2).

The low enthalpy value of the purified PG showed that the formation of the transition state or activated complex between the enzyme–substrate was very efficient. Moreover, as explained earlier by Muhammad *et al*. [22], the low ΔG^* value suggested a more spontaneous conversion of the transition complex (E–S) into products. Moreover, the

Table 2. Apparent Michaelis constants and thermodynamic parameters for polygalacturonic acid hydrolysis of purified PG from *Ecc* BR1.

Kinetic parameters		Thermodynamic parameters	
K_m [a]	0.67 mg/ml	ΔH^*	7.98 kJ/mol
V_{max}	2.38 Units	ΔS^*	−194.48 J/mol/K
k_{cat} [b]	21/sec	ΔG^* (free energy for activation)	68.86 kJ/mol
k_{cat}/K_m [c]	31.34	ΔG^*_{E-S} (free energy for substrate binding)	−1.04 kJ/mol
V_{max}/K_m [d]	3.55	ΔG^*_{E-T} (free energy for transition state formation)	−8.96 kJ/mol

[a]Michaelis constant for substrate affinity.
[b]Turnover number.
[c]Second-order rate constant.
[d]Catalytic efficiency.

low entropy value indicated that the transition complex (E−S) of the PG had less disorder. The feasibility and extent of the chemical reaction is best determined by measuring the change in the ΔG^* for substrate hydrolysis, (*i.e.*, the conversion of the E−S complex into products) [22]. Futhermore, the free energy for the activation of the substrate binding (ΔG^*_{E-S}) and free energy for the formation of the activation complex (ΔG^*_{E-T}) of the purified PG also reconfirmed that the enzyme had a high affinity towards polygalacturonic acid for hydrolysis and its spontaneous conversion into D-galacturonic acid (Table 2).

Thermal Stability Studies of Purified PG

Thermal stability represents the capability of an enzyme molecule to resist thermal unfolding in the absence of a substrate, whereas thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of a substrate. The stability of enzymes in this case is always judged by the residual activity [8].

To measure the kinetics of the thermal inactivation of the PG from *Ecc* BR1, the enzyme was incubated at 20−70°C. A pseudo-first-order plot was applied to determine the extent of the thermal inactivation (Fig. 3). The PG was found to be stable at 20°, 30°, 40°, and 50°C, with a half-life ($t_{1/2}$) of 426, 192.5, 126, and 99 min, respectively. When increasing the temperature further, the inactivation rate increased. At 60 and 70°C, the inactivation rate increased drastically, with a decrease in the half-life to 36 and 10 min, respectively.

Thermal inactivation of enzymes occurs in two steps: $N \leftrightarrow U^* \rightarrow I$, where N is the native enzyme, U^* is the unfolded inactive enzyme that can be reversibly refolded upon cooling, and I is the inactivated enzyme formed after prolonged exposure to heat and is thus irrecoverable upon cooling [34].

The inactivation curves for the purified PG from *Ecc* BR1 were not linear in the range of temperatures studied (Fig. 3). Rather, a biphasic nature was evident, indicating that the inactivation of the PG was a first-order process, commonly observed for enzyme inactivation [27]. Thus, the purified PG from Ecc BR1 was found to be thermostable, similar to the PGs from commercial preparations of pectinase CCM [25].

Thermal Deactivation of Purified PG with Different pHs

The extent of deactivation of an enzyme is measured by the deactivation rate, which is proportional to the active enzyme concentration, and thus K_d (the deactivation rate constant) is considered as a proportionality constant [23]. The deactivation rate and half-life were studied at 30°, 40°, 50°, and 60°C at pHs 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 (Fig. 4A and 4C). pH is one of the main factors affecting the tertiary and quaternary structures of a protein and enzyme. In many cases, the rate of deactivation depends on the pH of the enzyme solution [32]. In this study, a pH range from 4.0 to 8.0 was selected, since the PG was found to exhibit optimum activity at pH 5.0. As shown in Fig. 4A and 4C, at 40°C (optimum temperature), the PG remained stable for

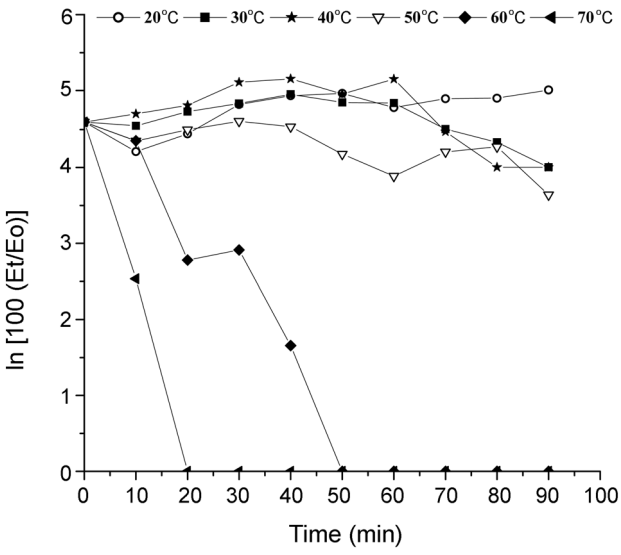

Fig. 3. Pseudo-first-order plot for irreversible thermal denaturation of purified PG from *Ecc* BR1.

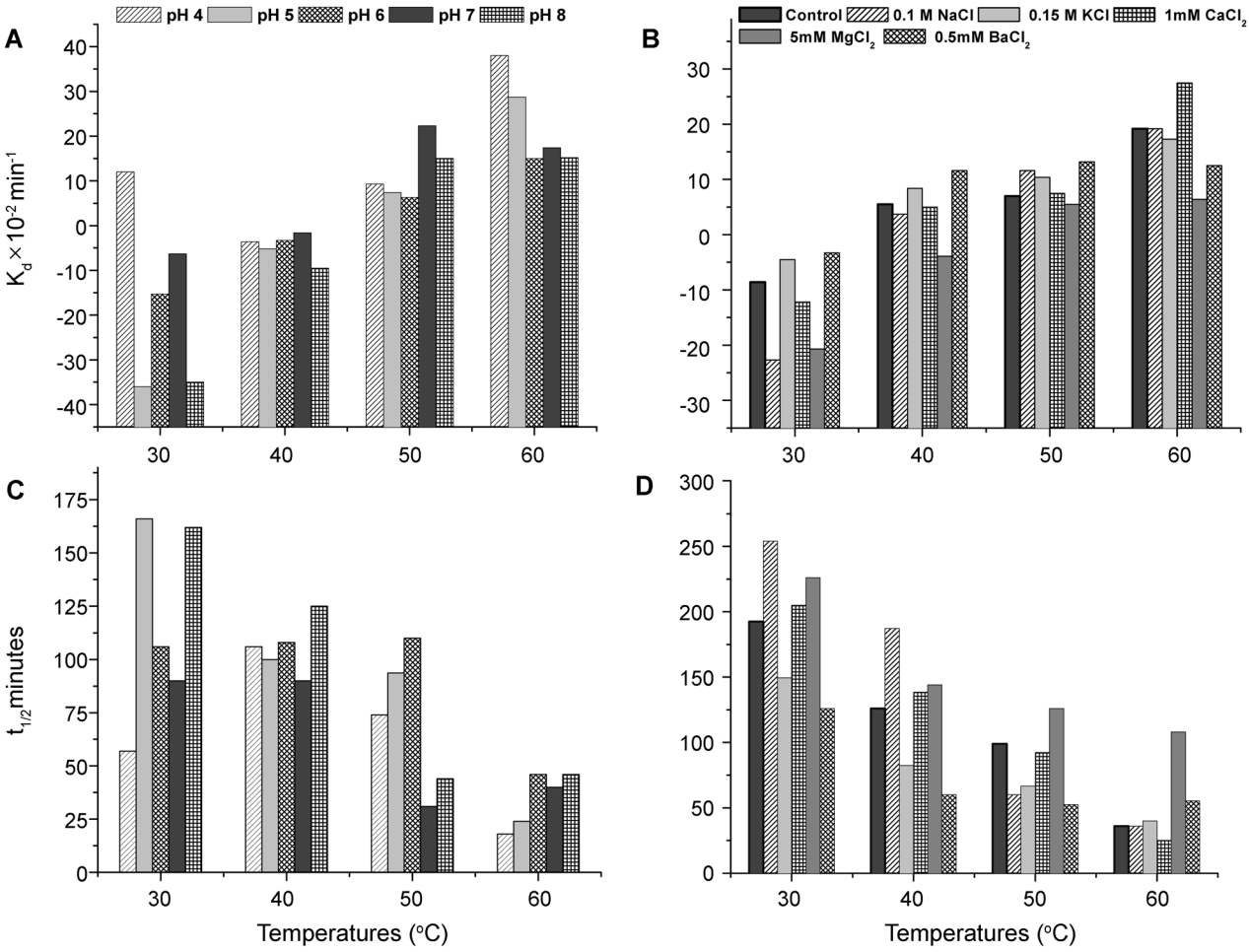

Fig. 4. Deactivation rate constant (K_d) (**A**, **C**) and half-life ($t_{1/2}$) (**B**, **D**) measurements of purified PG from *Ecc* BR1 with different pHs and salts.

all the pH conditions (pH 4.0–8.0), whereas at 60°C (higher than optimum temperature), the stability of the PG increased when the pH became more alkaline, where the order of stability for the various pH conditions was as follows: pH 8≥pH 6>pH 7>pH 5>pH 4 (Fig. 4A and 4C). At a higher pH, the folding of the PG possibly relaxed owing to changes in the balance of the electrostatic and hydrogen bonds, resulting in an increased stability [15, 32].

However, the half-lives ($t_{1/2}$) of the PG under different pH conditions did not increase uniformly as the pH became more alkaline; the only exception was at 60°C, where there was a steady increase in the $t_{1/2}$ (*viz*., 18, 24, 46, 40, and 46 min) when increasing the pH in the range of 4.0–8.0, respectively. In this study, at a temperature of 30°C, the $t_{1/2}$ of the purified PG was optimum (166 min) at pH 5.0 and lowest (57 min) at pH 4.0, whereas at a temperature of 40°C, the $t_{1/2}$ values were similar to those in the range of pH 4.0–7.0. Only at pH 8.0 was the half-life of the PG found to be greater (125 min) than that at a pH range of 4.0–7.0 (Fig. 4C). The increase in the half-life of the purified PG in the pH range of 4.0–8.0 with a temperature range of 30–60°C was similar to other reports on the half-lives of partially purified PG-I and PG-II from *A. niger* [23] and other enzyme chitinases of *Pantoea dispersa* [9].

Thermal Deactivation of Purified PG with Different Salts

The deactivation rate and half-lives of the purified PG were studied at a temperature range of 30–60°C in the presence of different salts. The deactivation rate increased when increasing the temperature; in other words, the half-life decreased when increasing the temperature. The addition of 0.1 M NaCl, 0.15 M KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, or 0.5 mM $BaCl_2$ to the purified PG reduced the deactivation rate and increased the half-life at all the temperatures studied (Fig. 4B and 4D).

The effectiveness of the various salts was in the following order: $MgCl_2$>$BaCl_2$>KCl>$CaCl_2$>NaCl, indicating that the effect of salt on the stability of the enzyme was related to the type of salt and its concentration. The stabilization of

the PG by salt may have been due to a reduction in unfavorable electrostatic repulsion, which led to reduced unfavorable electrostatic free energy. The stability of the PG was found to be optimum with $MgCl_2$, in which case lower K_d values were obtined for the PG with a temperature range of 30–60°C (Fig. 4B). Overall, the salts used in this study were found to increase the stability of the PG. Furthermore, the level of salt required to enhance the stability of the PG was within the range generally found in soils. The half-lives of the PG with the different salts were found to increase when compared with the PG without any salt, especially with $MgCl_2$, where the $t_{1/2}$ was higher at a temperature range of 30–60°C (Fig. 4D). These half-lives were significantly greater than those previously reported for PG-I and PG-II from *A. niger* [23].

Entropy Change During Thermal Deactivation of Purified PG with Different Salts and pHs

In addition to the deactivation study, an investigation of other thermodynamic parameters (ΔG^*, ΔH^*, ΔS^*, and deactivation energy) is also necessary to understand the behavior of molecules in different physiological conditions and the complex process of deactivation [23]. Negative entropy is generally found in biocatalytic systems owing to compaction of the enzyme molecule [5]. Accordingly, the transition state of the PG was ordered, as revealed by its negative ΔS^* value, which was comparatively lower for its thermal deactivation (Table 3). The change in entropy was calculated using the transition state theory according to Eq. (12). An enzyme is rendered more thermostable by stabilizing the native form with a noncovalent bond, including a hydrogen bond, salt bridge, or hydrophobic interaction, or by decreasing the entropy of unfolding [3]. Similar kinds of observation were noted with the addition of each salt and at every pH for the PG from *Ecc* BR1 (Table 3).

Table 3. Thermodynamic parameters measured during thermal deactivation of purified PG from *Ecc* BR1 with different salts, pHs, and temperature range of 30–60°C.

Enzyme system	ΔH^* (kJ/mol)	ΔS^* (J/mol K)	E (kJ/mol)
Polygalacturonase (PG)	42.60	−133.04	43.65
PG+0.1 M NaCl	73.08	−38.85	68.42
PG+0.15 M KCl	62.26	−69.56	30.04
PG+1 mM $CaCl_2$	75.62	−30.39	70.92
PG+5 mM $MgCl_2$	47.12	−126.59	89.53
PG+0.5 mM $BaCl_2$	55.93	−91.04	84.68
PG+pH 4	41.51	−136.38	25.87
PG+pH 5	68.50	−57.71	105.32
PG+pH 6	69.77	−53.13	135.47
PG+pH 7	83.57	−67.30	118.44
PG+pH 8	78.07	−25.11	170.00

In the presence of $MgCl_2$, the ΔS^* was lower (−126.59 J/mol K) than that with the other salts, although there was only a marginal difference in the ΔS^* (−133.04 J/mol K) for the PG in the absence of salt. The entropy change for the thermal inactivation of the PG in the presence of the different salts had the following order: $CaCl_2$>NaCl> KCl>$BaCl_2$>$MgCl_2$ (Table 3). The increased ΔS^* indicated an increase in the number of protein molecules in a transition activated state [23].

The PG at pH 4 had the lowest value for ΔS^* (−136.38 J/mol K) when compared to the other pH conditions. The entropy of the PG at the various pHs showed the following order: pH 4<pH 7<pH 5<pH 6<pH 8 (Table 3). The magnitude of the entropic term of the enzymatic reaction decreases as the enzyme stability increases [2]. For all the different pHs, the entropy changes were found to be negative (Table 3), which is consistent with a compaction of the reacting enzyme molecule, although such changes could also arise from the formation of charged particles and the associated gain and ordering of solvent molecules [5].

Enthalpy and Activation Energy Change of PG Deactivation with Different Salts and pHs

The enthalpy change (ΔH^*) and deactivation energy (E) of PG were estimated within a temperature range of 30–60°C in the presence of various salts and at different pHs. It has been reported that the enthalpy change of enzymes should be in a range of 20–150 kJ/mol during deactivation [2]. Here, the enthalpy value was calculated using Eq. (11). The enthalpy increased in the presence of salts with the following order: $MgCl_2$<$BaCl_2$<KCl<NaCl<$CaCl_2$ (Table 3). However, the enthalpy increase with the different pH conditions was random, although within a range of 20–150 kJ/mol. Thus, the ΔH^* of the PG deactivation remained within the reported range in the presence of each salt and at every pH, demonstrating the major involvement of enthalpic stabilization of the protein structure in temperature adaptation. The decrease of ΔH^* as the enzyme stability increased mainly reflected a decrease in the cooperation of inactivation and unfolding. For instance, a heat-labile enzyme denatures in a shorter temperature range, leading to a sharp slope for the Arrhenius plot and subsequently to a high activation energy E_a and ΔH^*. Such high cooperativity probably originates from the lower number of interactions required to disrupt the active conformation [2].

The enthalpies of the PG were moderately lower with all the salts tested at a temperature range of 30–60°C. The ΔH^* of the purified PG showed low values in the presence of $MgCl_2$ and $BaCl_2$ when compared with the other salts, indicating that salts allowed thermal stabilization of the enzyme. The enthalpy of the PG at the pH range (pH 5.0–8.0) tested showed fewer variations. The ΔH^* value was

lower (41.512 kJ/mol) at pH 4.0 than at the other pHs tested (Table 3).

The deactivation energy (*E*) was calculated from the slope of a linear plot of 1/T versus ln[K_d] using Eq. (15), as shown in Table 3. The deactivation energy of 170 kJ/mol was maximum at pH 8.0 for the PG and started decreasing towards an acidic pH range. The deactivation energy increased at an alkaline and neutral pH, suggesting that the enzyme required a higher amount of energy to deactivate at these pH conditions. The deactivation energy (*E*) increased for every condition in the following order : KCl<NaCl< $CaCl_2$<$BaCl_2$<$MgCl_2$, indicating that the divalent cationic salts forced the enzyme to require more energy for thermal deactivation when compared with the remaining monovalent cationic salts.

Free Energy Change of PG Deactivation with Different Salts and pHs

The Gibbs free energy (ΔG^*) measures the spontaneity of a reaction. In turn, this thermodynamic parameter measures the combination of changes in heat and entropy that occurs during a reaction. An increase in ΔS^* implies an increase in the number of protein molecules in a transition activated state, which in turn gives a lower value of ΔG^* [33]. The value of ΔG^* for the PG deactivation was calculated using Eq. 13 (Table 4). For the PG enzyme, the ΔG^* increased when increasing the temperature. However, this was comparatively higher than that previously reported for the partially purified PG from *A. niger* [23]. Iyer and Ananthanarayan [12] proposed that a kinetic analysis of enzyme inactivation mechanisms is of prime importance allowing for better control over biocatalyst use. In addition, the resistance of enzymes to thermal denaturation is due to the intrinsic contribution of the polypeptide chain (*i.e.*, hydrophobic interaction, hydrogen bonding, and ionic stabilization).

To assess the contribution of various salt ions to the thermal stabilization of the PG from *Ecc* BR1, the effects of different salts on the themal stability of the PG at its optimum activation concentration were studied at 30–60°C. The free energy of the PG marginally varied with each salt, indicating that the thermal stability of the PG was to some extent dependent on an interaction with the salt. The ΔG^* decreased in the following order: $MgCl_2$> $BaCl_2$>$CaCl_2$≥NaCl>KCl with an increase in temperature. Similar results were also obtained when varying the pH condition, where the ΔG^* decreased in the following order: pH 7>pH 5>pH 6>pH 8>pH 4, although the decrease in the ΔG^* was not as uniform as the pH became more alkaline (Table 4). The monovalent cations showed a lower ΔG^* when compared with the divalent cation used in this study. It has been reported that the mechanism by which salt affects enzyme stability may be explained by the effect of salt on the water structure and hence on the strength of the hydrophobic interaction [7].

In conclusion, the purified PG of *Ecc* BR1 is a catalytically efficient enzyme for polygalacturonic acid hydrolysis. Its ability to hydrolyze different types of pectic substrate, as well as its kinetic and thermodynamic parameters, makes the enzyme versatile and efficient for industrial application. The stability of the enzyme with different salts and pHs may also be a useful attribute to utilize when applied as an alternative commercial preparation. Enzyme deactivation is equally important to an industrial process; however, scant attention is given to such studies. Notwithstanding, these studies enhance our knowledge of the PG mechanisms, which may lead to the establishment of inhibition and interaction studies at a molecular level. The PG from *Ecc* BR1 could be a potential candidate for specific applications in the food, waste treatment, textile, and paper industries. It should not be ignored that the feasibility of obtaining a PG with novel industrial potential is higher from a new microbial strain not previously reported for this purpose (as in this case).

Table 4. ΔG^* values for thermal deactivation of purified PG from *Ecc* BR1 with different salts and pHs.

Enzyme system	ΔG^* (kJ/mol)			
	30°C	40°C	50°C	60°C
Polygalacturonase (PG)	83.00	84.23	85.56	86.10
PG+0.1 M NaCl	84.85	85.14	85.44	85.74
PG+0.15 M KCl	83.34	84.05	84.73	85.43
PG+1 mM $CaCl_2$	84.83	85.14	85.44	85.74
PG+5 mM $MgCl_2$	85.47	86.55	87.81	89.27
PG+0.5 mM $BaCl_2$	83.51	84.42	85.33	86.24
PG+pH 4	82.84	84.20	85.56	86.93
PG+pH 5	85.99	86.56	87.14	87.71
PG+pH 6	85.87	86.40	86.93	87.46
PG+pH 7	103.96	104.04	105.31	105.98
PG+pH 8	85.68	85.93	86.18	86.43

Acknowledgment

Author M.V.B. is grateful to the University Grants Commission (New Delhi, India) for a meritorious student research fellowship (UGC Sanction No. F.4-1/2006/XI plans/BSR).

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