Effect of Additives on Kinetic Thermal Stability of Polygalacturonase II from *Aspergillus carbonarius*: Mechanism of Stabilization by Sucrose

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The thermal inactivation of polygalacturonase (PG II; M_w = 42 kDa) from *Aspergillus carbonarius* at optimum pH of the enzyme activity followed first-order kinetics both in the presence and in the absence of additives. The enzyme was stable below its optimum pH. Sodium chloride enhanced the thermal stability, the midpoint of thermal stability (T_m), shifted by 6 °C, and the half-life at T_m increased by 68-fold. Except for ethylene glycol, the other polyhydric alcohols enhanced thermal stability in a concentration-dependent manner; the stabilizing effect by polyhydric alcohols increased with increase in hydroxyl content of the polyol. The activation enthalpy increased in the presence of 1 M sucrose and 1 M NaCl from 77 to 116 kcal mol^{-1}; activation entropy increased from 169 to 290 cal deg^{-1} mol^{-1}. The net free energy change of stabilization in the presence of stabilizers was 3−4 kcal mol^{-1}. The thermal inactivation of the enzyme involved conformational changes and unfolding of the enzyme molecule; the major secondary structural element, β structure, decreased and the tryptophan residues were exposed to solvent. Stabilization of enzyme by sucrose was by preventing unfolding of the enzyme.

Keywords: *Additives; polyhydric alcohols; polygalacturonase; pectinases; thermal stability; mechanism of stabilization*

INTRODUCTION

Polygalacturonase (PG, EC 3.2.1.15) is a member of pectinase family that acts on α-1−4 linkages of polygalacturonic acid (PGA) in pectin, a cementing substance in plant cell wall, and causes structural degradation (Kertesz, 1951). Its tissue-softening property is commercially exploited in the food industry in the extraction and clarification of fruit juices (Pilnick and Voragen, 1990). Bacteria, fungi, and higher plants produce multiple forms of polygalacturonase. *Aspergillus carbonarius* secretes PGs that are active and stable in acid pH range (Sreekantaih et al., 1975). Three multiple forms of PG differing in their physicochemical properties have been purified and characterized (Devi and Rao, 1996). The major form, PG II, which has molecular weight (M_w) of 42 kDa, accounting for 60% of total PG activity, has a specific activity of 7000 units (mg of protein)$^{-1}$, the highest reported so far.

Many fungal PGs are thermolabile and become irreversibly inactivated by 60 °C with a few exceptions such as *Penicillium* (Gillespie et al., 1990), *Rhizopus* (Ros et al., 1993), and *Sclerotinia* (Archer and Fielding, 1975). These studies are limited to measuring the remaining activity after incubation at higher temperatures for a specific period of time.

Biotechnological applications of enzymes are limited because of their sensitivity toward operational parameters such as higher temperature, pH, and salt concentrations. Enhanced thermal stability is a desirable feature for economic viability of enzymatic processes (Wasserman, 1984). One of the approaches for enhanced thermostabilization of enzymes is to alter the microenvironment using additives (Schmid, 1979).

Modification of the microenvironment of enzymes using salts (von Hippel and Sleich, 1969), organic solvents (Biringer and Fink, 1982), and polyhydric alcohols (Arakawa and Timasheff, 1982; Back et al., 1979; DeCordt et al., 1994; Gerlsma, 1970) have been tried with considerable success for understanding the mechanism of thermal inactivation. The use of polyhydric alcohols could be a valuable method for the thermostabilization where immobilization or covalent modification is not feasible (Gray, 1988). Thermal stability of an enzyme is greatly influenced by the solvent structure. Additives that modify the solvent structure such as sugars, or polyhydric alcohols, act as stabilizing agents. The stabilizing effect of the additive is not valid in all cases but would depend on the nature of the enzyme studied, on its hydrophilic/hydrophobic character, and on the degree of its interaction with the additives (Ye et al., 1988).

In the present investigation for the first time we have made a systematic study of the stabilizing effect of various additives on PG II from *A. carbonarius* in an attempt to protect the enzyme against thermal inactivation and to understand the mechanism of thermal inactivation. Of the two approaches for following stability, because of irreversible thermal inactivation of the enzyme (Devi and Rao, 1996), the thermodynamic approach was not possible; hence, a kinetic approach was used in the present study for stability measurements. Measuring the residual activity as a function of time and determining the kinetic rate constants is a

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more convenient approach to study the effect of solvents on protein stability. On this basis, an Arrhenius plot can be derived and the thermodynamic parameters such as free energy (ΔG^*), enthalpy (ΔH^*), and entropy (ΔS^*) can be calculated. These data are used to assess the extent of protein stabilization and the influence of the additive on protein conformation. Our investigation has suggested that PG II could be stabilized by modifying the microenvironment of the enzyme; the additives prevented conformational changes during thermal inactivation.

MATERIALS AND METHODS

PG II ($M_w = 42000$) with specific activity of 7000 units/mg was purified to electrophoretic homogeneity from the commercial enzyme preparation of fungal source *A. carbonarius* as reported previously (Devi and Rao, 1996). The commercial enzyme preparation was from Triton Chemicals, Mysore, India.

Substrate polygalacturonic acid (PGA), galacturonic acid, citrus pectin, different polyhydric alcohols, poly(ethylene glycol) (PEG) of different molecular weights, and acrylamide were from Aldrich Chemical Co. Inc. (Milwaukee, WI). Freshly distilled monohydric alcohols were used. All other salts and buffer salts were purchased from Merck, India, and Qualigens (Glaxo), India. They were of analytical reagent grade.

Enzyme Activity Assay. The activity of enzyme was quantified by estimating the reducing equivalents liberated from PGA, according to Somogyi's alkaline copper reduction method (Ashwell, 1957). The assay mixture consisted of 5 mL of 0.5% PGA and 0.1 mL of PG II (1–2 μg) in 0.1 M acetate buffer, pH 4.3. At the end of incubation period of 5 min at 50° C, 0.1 mL of reaction mixture was withdrawn and liberated reducing equivalents from PGA were estimated. Galacturonic acid was used as standard reference.

Unless otherwise mentioned, PG II was taken at a concentration of 10 μg/mL in 0.1 M acetate buffer, pH 4.3, containing 0.1 M sodium chloride for all measurements. The enzyme was stored at 4 °C in the above-mentioned buffer. There was no loss in the enzyme activity a period of 6–8 weeks.

Thermal Inactivation Measurements. PG II in the presence and absence of specific additives at desired concentrations was incubated at different temperatures between 25 and 60 °C for 15 min. A 0.1 mL sample was drawn at the end of a specified time interval and assayed for remaining activity as described above. Activity of the unincubated sample was taken as 100%, and the percent apparent remaining activity of heated samples were calculated. The midpoint of thermal inactivation temperature (T_m) is the temperature at which 50% of activity remained. Kinetics of thermal inactivation was carried out at 46 °C (T_m) for up to 5 h. PG II in different concentrations of additives (polyols, salts, PEG, monohydric alcohols, and 1% citrus pectin) was incubated for different time intervals. For experiments in the presence of various salts, PG II in 0.1 M acetate buffer pH free from sodium chloride was used. A 0.1 mL aliquot of each sample was withdrawn at various intervals throughout the incubation time and assayed immediately under standard conditions. Percentage relative catalytic activity remaining was determined for each time point; that is, the activity at time t as a percentage of that at time 0. The apparent half-lives (defined as the time required for PG II activity to decline to 50% of its initial value) were estimated by inspection of plots of log percent remaining activity versus time. Inactivation rate constants (k_r) were obtained from the slopes of the plots of linear regression of logarithm of relative percent activity versus time.

Activation Energy Measurements. The enzyme PG II in the presence of 1 M sucrose containing 1 M sodium chloride or 2 M sodium chloride alone and in the absence of any of the above was incubated at different temperatures (37–57 °C) for various time periods, and kinetics of thermal inactivation was measured.

Calculation of Activation Parameters. The temperature dependence of rate constant for inactivation was analyzed according to an Arrhenius plot. The activation energy (E_a) was obtained from the slope of the Arrhenius plot (regression of logarithm of reaction rate constants versus reciprocal of absolute temperature). Activation enthalpy (ΔH^*) for each temperature was calculated according to

$$\Delta H^* = E_a - RT \tag{1}$$

The values for free energy of inactivation (ΔG^*) at different temperatures are calculated from the first-order rate constant of inactivation process by

$$\Delta G^* = -RT \ln(k_r h/kT) \tag{2}$$

where k_r is the inactivation rate constant, h is Planck's constant, k is the Boltzmann constant, R is the universal gas constant, and T is temperature in kelvin. Activation entropy (ΔS^*) was calculated as

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \tag{3}$$

Fluorescence Measurements. Fluorescence measurements were performed on a Shimadzu RF 5000 spectrofluorophotometer. PG II having an absorption of 0.07–0.08 at 280 nm (50–60 μg mL^{-1}) was used. Protein solution was taken in a 1 cm path length quartz cuvette, excitation was at 285 nm with 5 nm slit width, and emission was recorded at 336 nm with 10 nm slit width. Protein fluorescence was measured in the range 20–60 °C both in the presence and in the absence of 1 M sucrose. Desired temperature was obtained by cirulating water from a thermostated water bath and allowing 10 min for thermal equilibration.

The enzyme fluorescence from tryptophan residues was quenched by progressive addition of small aliquots (10 μL) of nonionic quencher [acrylamide (2 M)] and ionic quencher [potassium iodide (2 M)] containing 0.1 mM sodium thiosulfate. The absorbance of potassium iodide at the excitation wavelength was not detectable; thus, only dilution correction was given. The inner filter effect due to the absorption of acrylamide at the excitation wavelength (295 nm) was corrected using the equation (Eftink and Ghiron, 1976)

$$F_{obs} = (F_{corr}) \times 10^{A/2} \tag{4}$$

where F_{obs} is the observed fluorescence, F_{corr} the corrected fluorescence, and A the absorbance at 295 nm of added acrylamide. The quenching data were analyzed according to the Stern–Volmer (Lehrer, 1971)

$$F_0/F = 1 + K_{SV}[Q] \tag{5}$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher respectively, [Q] is the quencher concentration, and K_{SV} is the dynamic quenching constant of the system. The fractional accessibility value (fa) and modified Stern–Volmer constant (K'_{SV}) were determined using (Lehrer and Leavis, 1978)

$$F_0/\Delta F = 1/(fa)K'_{SV}[Q] + 1/(fa) \tag{6}$$

where ΔF is equal to $F_0 - F$ and fa is maximum fractional accessibility of protein fluorescence.

Circular Dichroism (CD) Measurements. CD measurements were made on a JASCO-J20C automatic recording spectropolarimeter fitted with a xenon lamp. Far-UV spectra were recorded at 30 °C between 200 and 260 nm using a 1 mm path length quartz cell. PG II of 0.34 mg/mL concentration was taken for all measurements. The CD data were expressed as mean residue molecular ellipticity [Θ] in deg cm^2 dmol^{-1} by using a value of 115 for mean residue weight (MRW). The secondary structure content was analyzed by the computer program CDPROT (Menendez-Arias et al., 1988).
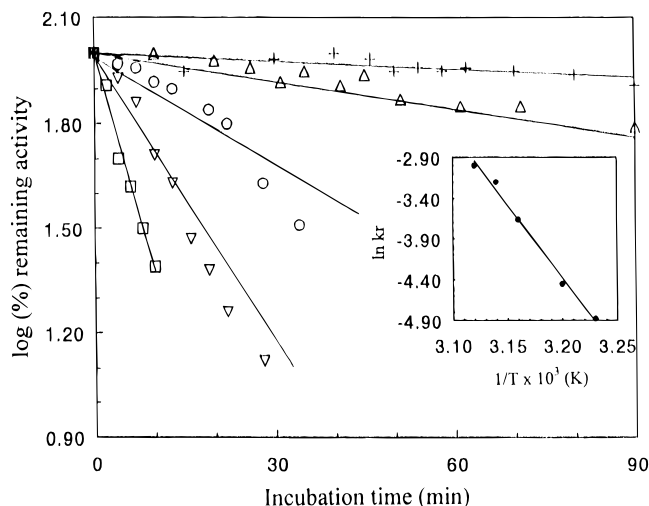
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Figure 1. Kinetics of inactivation at different incubation temperatures as shown by a semilogarithmic plot for PG II in 0.1 M acetate buffer pH 4.3: (+) 37 °C; (△) 40 °C; (○) 44 °C; (▽) 46 °C; (□) 48 °C; (inset) Arrhenius plot.

Measurement of Kinetic Parameters. The K_m and V_{max} of PG II in the presence and absence of 1 M sucrose were determined with the substrate PGA in the concentration range 0.2–1%. Activity was assayed as mentioned above.

All of the kinetic and spectroscopic data reported are an average of three sets of measurements.

RESULTS

The kinetics of thermal inactivation of the native enzyme, in the temperature range 37–48 °C at pH 4.3, the optimum pH for enzyme activity, was measured. The logarithm of relative activity versus time is shown in Figure 1. At all of the temperatures studied, the inactivation can be explained as a single-exponential decay, indicating that inactivation followed first-order kinetics. The Arrhenius plot was linear in the temperature range studied. The activation parameters such as free energy, enthalpy, and entropy are given in Table 1.

Effect of Various Salts on Kinetics of Thermal Inactivation. The thermal inactivation of PG II was measured in the presence of various concentrations of sodium chloride at 46 °C. With increase in the concentration of sodium chloride the half-life of the enzyme increased (Figure 2) and the rate constants decreased. The extrapolation of rate constant as a function of sodium chloride concentration (Figure 2, inset) suggested, at 2.65 M concentration, the rate constant was 8.3×10^{-7} s^{-1} and half-life was 229 h. In the presence of 2 M sodium chloride T_m shifted from 46 to 52 °C (data not shown).

Effect of other salts such as KCl, MgCl$_2$, CaCl$_2$, and NaBr on the kinetics of thermal inactivation were followed by measuring rate constant at 46 °C. NaBr had no effect on the rate constant; KCl and MgCl$_2$ decreased the rate constants. CaCl$_2$, in contrast to the above salts, inactivated the enzyme at 0.1 M concentration. This would suggest that stabilizing due to salt is ion specific.

Stabilization by Polyhydric Alcohols. The effect of various polyhydric alcohols such as ethylene glycol, glycerol, erythritol, xylitol, D-sorbitol, and sucrose on the thermal stability of the enzyme was followed by measuring the T_m, half-life, and rate constants of thermal inactivation. The T_m measurements were made in the

Table 1. Activation Parameters of PG II in the Presence of Additives

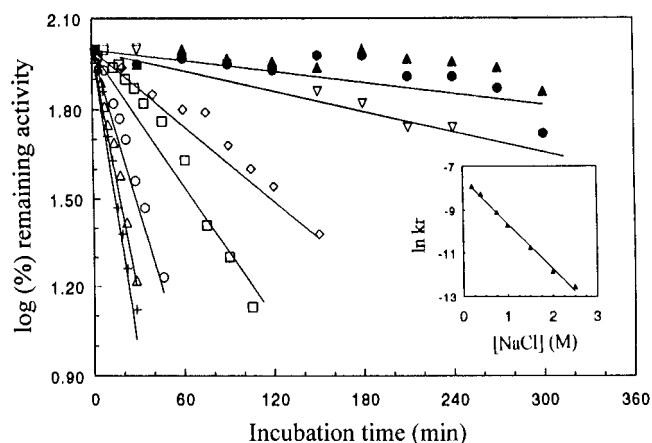| incubn temp (°C)	half-life (min)	rate constant, $k_r \times 10^{-4}$ (s^{-1})	ΔG^* (kcal mol^{-1})	ΔH^* (kcal mol^{-1})	ΔS^* (cal^{-1} deg^{-1} mol^{-1})
		Control			
37		0.3 ± 0.02	24.6	77.4	170.0
40	141	0.8 ± 0.04	24.2	77.4	170.0
44	23	5.1 ± 0.25	23.4	77.4	170.0
46	8	14.4 ± 0.4	22.9	77.4	171.0
48	5	23.0 ± 0.7	22.7	77.4	171.0
		E_a = 78 kcal mol^{-1}			
		PG II in the Presence of 1 M Sucrose Containing 1 M NaCl			
50		0.25 ± 0.02	25.8	116.1	280
52	118	1.0 ± 0.05	24.9	16.1	281
53	87	1.30 ± 0.07	24.8	161.1	280
55	14	8.20 ± 0.25	23.8	116.1	281
56	14	8.20 ± 0.40	23.8	116.1	281
57	8	14.4 ± 0.70	23.6	116.1	280
		E_a = 116.7 kcal mol^{-1}			
		PG II in the Presence of 2 M NaCl			
48	110	1.04 ± 0.05	24.6	77.5	165.0
50	50	2.30 ± 0.20	24.2	77.5	165.0
52	18	6.40 ± 0.30	23.7	77.5	165.5
54	12	9.60 ± 0.30	23.7	77.5	165.0
56	3	38.5 ± 1.10	22.9	77.5	166.0
		E_a = 78.2 kcal mol^{-1}			

Figure 2. Semilogarithmic plot of kinetics of thermal inactivation (46 °C) of PG II in 0.1 M acetate buffer, pH 4.3, containing different concentrations of NaCl: (+) control; (△) 0.2 M; (○) 0.4 M; (□) 0.75 M; (◇) 1.0 M; (▽) 1.5 M; (●) 2 M; (▲) 2.5 M; (inset) semilog plot of ln k vs NaCl concentration.

presence of 1 M concentration of the added polyhydric alcohol. The enzyme gets completely inactivated irreversibly at 50 °C in the absence of any additives, the T_m being at 46° C. With the exception of ethylene glycol, polyhydric alcohols increased the T_m and half-life of PG II as a function of hydroxyl content in the polyhydric alcohol. Sucrose shifted the T_m by 6 °C. Sucrose and sorbitol were very effective in stabilizing PG II. The various rate constants and half-lives in the presence of polyhydric alcohols as a function of concentration are given in Table 2. In the case of sucrose, kinetic plots were nonlinear at higher concentrations of sucrose beyond 360 min, and their rate constants were obtained from the initial linear portions of curve. The magnitude of standard free energy of inactivation reflects the order of relative stabilization effectiveness of the various additives as determined at a single step.

Mechanism of Stabilization. To understand the mechanism of thermal stabilization, the kinetic param-

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Table 2. Half-Life at 46 °C and Rate Constants of PG II in the Presence of Salts and Polyol

Sl no.	additive	concn (M)	half-life (min)	rate constant, $k_r \times 10^{-4}$ (s^{-1})	ΔG^* (kcal mol^{-1})
enzyme	control		8	14.4 ± 0.7	22.9
	salts				
I	MgCl$_2$	1	34	3.5 ± 0.24	23.8
II	KCl	1	44	2.5 ± 0.2	24.0
III	NaCl	1	82	1.4 ± 0.1	24.3
		2	722	0.16 ± 0.02	25.7
polyols					
1	ethylene glycol	1	14	8.3 ± 0.4	23.2
		2	11	10.1 ± 0.4	23.1
		3	6	19.1 ± 0.9	22.7
		4	4	28.8 ± 0.9	22.4
II	glycerol	1	12	9.7 ± 0.5	23.1
		2	25	4.6 ± 0.2	23.6
		3	25	4.6 ± 0.2	23.6
III	erythritol	0.5	16	7.1 ± 0.14	23.3
		1	42	2.8 ± 0.14	23.9
		2	250	0.46 ± 0.02	25.0
		3	1283	0.09 ± 0.01	26.1
IV	xylitol	0.5	22	5.3 ± 0.3	23.5
		1	110	1.04 ± 0.05	24.5
		2	1283	0.09 ± 0.01	26.1
		3	1283	0.09 ± 0.01	26.1
V	sorbitol	0.5	29	4.1 ± 0.2	23.7
		1	167	0.69 ± 0.02	24.8
		2	1283	0.09 ± 0.01	26.1
		3	1283	0.09 ± 0.01	26.1
VI	sucrose	0.5	42	2.8 ± 0.14	23.9
		1[a]	1020	0.11 ± 0.01	26.0
		1.5[a]	1650	0.07 ± 0.01	26.2
		2[a]	1650	0.07 ± 0.01	26.2
VII	sucrose NaCl	1 1	167.4[b]	0.0115 ± 0.001	27.4

[a] Rate constants and half-lives were calculated from the initial linear portions of inactivation kinetic plots. [b] Hours.

eters such as activation energy, entropy, and free energy of thermal inactivation were measured in the presence of 1 M sucrose and 1 M sodium chloride and in the presence of 2 M sodium chloride alone. In both systems inactivation kinetics followed first order. Various activation parameters are given in Table 1. The activation energy of PG II increased from 77 to 116 kcal mol^{-1} in 1 M sucrose containing 1 M sodium chloride. The entropy of the system changed from 169 to 290 cal deg^{-1} mol^{-1}. The net free energy change of stabilization was 4.5 kcal mol^{-1}. In contrast, 2 M sodium chloride alone had no effect on activation energy and activation entropy, but net free energy change of stabilization was 2.7 kcal mol^{-1} (Figure 3; Table 2).

Effect of Sucrose on K_m and V_{max}. To estimate the affinity of sucrose to enzyme, kinetic parameters such as the Michaelis−Menten constant, K_m, and velocity maximum, V_{max}, were determined in the presence of 1 M sucrose. Both in the presence and in the absence of 1 M sucrose, K_m (PGA) and V_{max} (PGA) (μmol min^{-1}) were 2% and 40, respectively. Thus, sucrose had no effect on the K_m, V_{max}, and V_{max}/K_m ratio.

Spectroscopic Measurements. The native enzyme has fluorescence emission maximum at 336 nm. Upon heating, with the increase in temperature there was a decrease in fluorescence intensity and a shift toward red (340 nm). The fluorescence intensity and emission maximum could not be regained after cooling from 50 to 25 °C, suggesting irreversible changes. The loss in activity and decrease in relative fluorescence intensity as a function of temperature are shown in Figure 4.
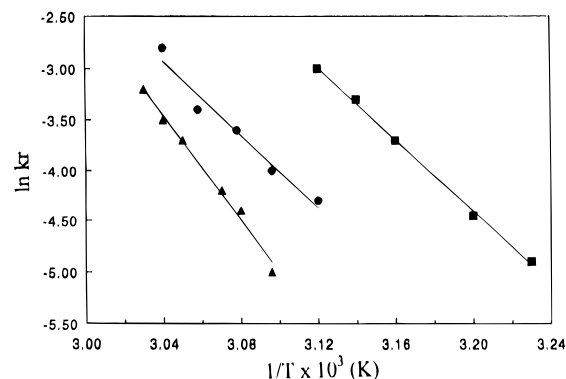

Figure 3. Arrhenius plot of thermal inactivation of PG II: (■) control; (●) in the presence of 2 M NaCl; (▲) in the presence of 1 M sucrose containing 1 M NaCl.
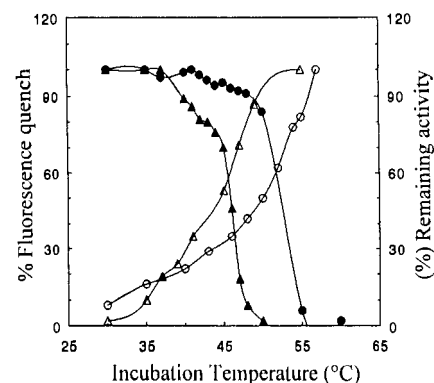

Figure 4. Effect of temperature on fluorescence intensity and enzyme activity: enzyme activity, (▲) control, (●) in the presence of 1 M sucrose (○, △); fluorescence quenching, percent quench was calculated as $[(F_0 - F)/F_0] \times 100$, where F_0 and F are RFI of unheated and heated samples, respectively.

Both had the same T_m; the overlap of loss in enzyme activity and decrease in fluorescence intensity is overwhelming.

Addition of 1 M sucrose had negligible effect on the fluorescence emission maximum and relative fluorescence intensity. The decrease in relative fluorescence intensity and loss in activity in the presence of 1 M sucrose as a function of temperature is shown in Figure 4. The T_m shifted by 6 °C. The shift in T_m measured as a decrease in either enzyme activity or relative fluorescence intensity was similar. The percent fluorescence quench rather linearly increased with temperature, indicating that the enzyme conformation is somewhat more easily lost than enzyme activity. In either case there was irreversible loss of activity with only a shift in the temperature of thermal inactivation in the presence of 1 M sucrose.

To quantitate subtle changes in the conformation, the quenching of fluorescence by acrylamide and potassium iodide was measured before and after heat inactivation. The Stern−Volmer plots and modified Stern−Volmer plots were linear in either of the cases. The Stern−Volmer constant (K_{SV}), modified Stern−Volmer constant (K'_{SV}), and fractional accessibility values (fa) for both of the quenchers before and after thermal inactivation are given in Table 3. In the case of quenching of fluorescence by acrylamide the K_{SV} and fa values did not change. There were significant changes in K_{SV}, K'_{SV}, and fa values in the case of potassium iodide for heat-inactivated enzyme. The K_{SV} constant increased from 0.65 to 2.2 and the fa value increased from 0.17 to 0.44,

Table 3. Fluorescence Quenching Constants of PG II and Heat-Inactivated Enzyme

sample	acrylamide			potassium iodide		
	K_{SV}	K'_{SV}	fa	K_{SV}	K'_{SV}	fa
control	2.9 ± 0.23	5.4 ± 0.4	0.68 ± 0.05	0.7 ± 0.05	6.2 ± 0.5	0.17 ± 0.01
heated	2.7 ± 0.23	4.1 ± 0.4	0.79 ± 0.06	2.2 ± 0.18	10.0 ± 0.7	0.44 ± 0.04
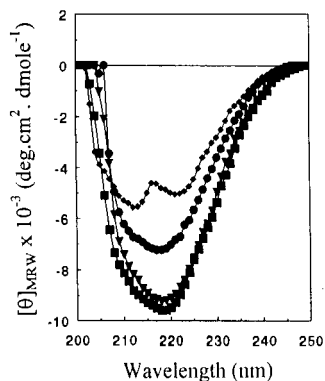
Figure 5. Effect of temperature on secondary structure of PG II: (■) control; (◆) control heated; (▼) in the presence of 1 M sucrose; (●) heated in the presence of 1 M sucrose.

suggesting an increase in exposure of tryptophan fluorophores due to unfolding.

CD Measurements. To quantitate the changes in the secondary structure due to heating, the far-UV CD spectra of native enzyme, enzyme heated to 50 °C, enzyme in the presence of 1 M sucrose, and enzyme heated to 50 °C in the presence of sucrose were measured and are shown in Figure 5. The native structure of the enzyme is characterized by a minima at 218 nm. Addition of 1 M sucrose had no effect on the amplitude of this band before thermal inactivation. Due to thermal inactivation of the enzyme in the absence of sucrose, there was a decrease in the ellipticity values below 260 nm.

There were two minima, one at 223 nm and the other at 211 nm. Addition of sucrose prevented the loss in the secondary structure (curve 4 in Figure 5).

DISCUSSION

The stability of an enzyme arises due to a delicate balance of a large array of noncovalent interactions between amino acid residues and with solvent and cosolvent molecules around the protein. Depending on the nature and concentration of the additives, they affect the protein conformation in three ways: (1) screening effect, where the electrostatic repulsion between similarly charged groups of proteins is reduced by cosolvent ions; (2) solvophobic effect, where ion pair formation occurs, favoring protein folding; and (3) modification of water structure leading to hydrophobic interactions in proteins. In this paper, we report the thermal inactivation of PG and effect of additives on this process.

Preliminary experiments on the effect of protein concentration on thermal inactivation show the inactivation process is independent of concentration up to 0.5 mg mL^{-1}. The kinetics of inactivation of PG II either in the presence or in the absence of additives and at all temperatures followed first-order kinetics, implying similar mechanisms being operative in the thermal inactivation process. Over the temperature range studied the activation parameters ΔH^* and ΔS^* changed but ΔG^* decreased by 1.9 kcal mol^{-1} with increase in temperature; also, the small value of ΔG^* (22.9 kcal

mol^{-1}) at 46 °C, under optimal conditions of activity, is indicative of the labile nature. The enzyme is marginally stable. Monohydric alcohols, such as methanol, ethanol, propanol, and butanol, and PEG of differing molecular weights from 300 to 2000 decreased the thermal stability of the enzyme (data not shown), which suggested that hydrophobic interactions contribute to the thermal stability of the enzyme. The increase in half-life and rate constants and activational energy (ΔH^*) in the presence of 1 M sucrose suggested that the stability observed in polyhydric alcohols could be conformational in origin. The activation entropy changed from 169 to 290 cal deg^{-1} mol^{-1}, which can be explained in terms of increased ordered or compact structure thus favoring intramolecular stabilizing force. Whereas in the case of NaCl the stabilization effect may be due to the effect on solvent structure, the ΔS^* value does not change. Activational energy for PG II in buffer and in the presence of 2 M NaCl is the same, that is, 78 kcal mol^{-1}, and that for sucrose and NaCl is more, that is, 117 kcal mol^{-1}, pointing to different mechanisms of stabilization. Saturation concentration of NaCl confered good thermal protection with a small increase of 2.7 kcal mol^{-1} in ΔG^*; hence, NaCl being a lyotrophic salt enhances protein stability by its effect on water structure. Thus, the increased values of ΔG^*, $\Delta S,^*$ and ΔH^* in the presence of 1 M sucrose and 1 M sodium chloride could be due to the combined effect on the conformation of protein and on solvent structure.

The loss in enzyme activity and the unfolding of the protein molecule as reflected in the decrease of fluorescence intensity over the temperature range studied are quite overlapping, suggesting unfolding of the protein molecule leads to irreversible inactivation. The shift in T_m is reflected in both activity and fluorescence measurements. The CD measurements and fluorescence changes were indicative of unfolding of the protein molecule and decrease in β structure content.

The major structural element in PG II was parallel β structure (Devi and Rao, 1998). The thermal inactivation led to a decrease in β structure with a concomitant increase in aperiodic structure. Both CD and fluorescence measurements suggested an irreversible unfolding of the molecule due to heating. The Stern–Volmer constants and fractional accessibility values for potassium iodide suggested partial exposure of fluorophores due to heating. Sucrose prevented conformational changes in the molecule.

The effect of salts such as NaCl on the increased thermal stability in acidic pH is indicative of the role of ionic interactions in thermal stability. It is interesting that although *A. carbonarius* is not a halophile, still the enzyme was stable in high salt concentrations. One percent citrus pectin (a substrate analogue) and high MW PEG, which contribute to the viscosity, do not affect the thermal stability of PG II, suggesting viscosity per se may not contribute to thermal stability.

The general propose scheme of thermal inactivation of enzymes is N ⇌ U → D (Klibanov, 1983). The single slope of Arrhenius plots indicates that thermal inactivation reaction for PG is a simple biochemical reaction;

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the attainment of transition state complex involves a single state process. In the case of PG II by the addition of polyhydric alcohol one can shift the equilibrium toward the native state, leading to themostabilization. The mechanism by which sucrose stabilizes is by preventing the unfolding without involving any specific interactions of sucrose with PG II. The ratio of V_{max} to K_m of PG II in the presence of sucrose confirmed this.

Thus, it is the unfolding of the enzyme molecule that leads to thermal inactivation of PG II, which could be prevented by the addition of polyhydric alcohols. By alteration in the microenvironmnent, one could enhance thermal stability and use of additive could be a versatile method for thermostabilization of enzymes mainly in enhancing the storage life of enzymes.

CONCLUSION

A systematic study on the thermal stability of polygalacturonases was made.

1. PGs from *A. carbonarius* are thermolabile.

2. There is an enhancement of stability ($\sim3-4$ kcal mol^{-1}) by additives such as NaCl and sucrose. The mechanisms of stabilization by NaCl and polyol are different.

3. In the case of PG II, the thermal inactivation is due to loss in secondary structure and exposure of tryptophan residues to the solvent resulting in unfolding of the molecule. This could be attributed to relatively unstable β structure, which constitutes the major structural element.

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