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ARTICLE

Effect of Cosolvents on the Structural Stability of Endoglucanase from *Aspergillus aculeatus*

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The effects of cosolvents such as sucrose, glycerol, and sorbitol on endoglucanase have been studied by activity, circular dichroic spectroscopy, fluorescence, and apparent thermal transition temperature measurements. The endoglucanase activity increased by 4-fold at 40% cosolvent concentration under optimum conditions. The endoglucanase lost 50% of its activity when exposed to 90 °C for more than 30 min (1 h). In the presence of cosolvents, it maintained its original activity and native conformation as indicated by far UV-CD at 70 °C. The app T_m increased from a control value of 57 °C to a value of 66 °C in the presence of 40% sucrose. The partial specific volume of endoglucanase was 0.723 mL/g in sodium acetate buffer. The preferential interaction parameters were negative in all cosolvents, and the maximum hydration of the protein was observed in 40% glycerol where the preferential interaction parameter was −0.126 g/g. The thermal stability of endoglucanase increased in the presence of cosolvents.

KEYWORDS: Endoglucanase; cosolvents; stabilization; conformation; preferential interaction

INTRODUCTION

Cellulose, a linear polymer of glucosyl units linked by β-1,4-linkages, constitutes most of the organic material on the surface of the earth. There is enormous production and degradation of cellulose in the biosphere. Because of the energy crisis in the present scenario, cellulose is considered an important source of renewable energy. Cellulases participate in the natural, ecological recycling of plant materials and are acquiring increasing commercial significance for use in detergents, foods, and paper. Many bacteria and fungi produce enzymes to break this complex substrate. Microbial cellulases have attracted considerable research and commercial interest due to their enormous potential applications in biotechnology and industry (*1*). Many cellulolytic microorganisms, especially fungi, produce extracellular cellulolytic systems consisting of endoglucanase (endo-1,4-β-D-glucanase; EC 3.2.1.4), exoglucanase (exo-1,4-β-D-glucanase; EC 3.2.1.91), and β-glucosidase (cellobiase; EC 3.2.1.21). Endoglucanase is the major component produced by *Aspergillus* for cellulose hydrolysis. Endoglucanases preferentially cleave the internal glycosidic bonds of cellulosic chains and act synergistically with exoglucanase and β-glucosidase during solubilization of crystalline cellulose (*2*, *3*). Endoglucanases play an important role in increasing the yield of fruit juices and beer filtrations and improving the nutritive quality of bakery foods and animal feeds. For these applications, it is necessary to have a wide range of endoglucanases with varying pH values and temperature stabilities. Protein stability is an important factor that determines its commercial applications.

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All of the solvent additives like sugars (sucrose) and polyhydric alcohols (glycerol and sorbitol) are known as cosolvents. All of these cosolvents stabilize globular proteins, having a common feature of preferentially hydrating proteins based on the preferential exclusion from the surface of the native protein at 20 °C (*4*). The use of enzymes in organic solvents has extended their practical applications and allowed the synthesis of biologically active enatiomers that are difficult to obtain with conventional chemical catalysts. The solvent influences the catalytic properties and stability of the enzyme to a large extent. Protein molecules in aqueous solutions are surrounded by a hydration shell, which is composed of water molecules attached to the protein surface. If an organic solvent is present, the solvent molecules tend to displace the water molecules both in the hydration shell and in the interior of the protein, thereby distorting the interactions responsible for maintaining the native conformation of the enzymes (*5*, *6*).

In the case of sugars, the stabilizing effect is due to the effect of the surface tension of water, and it is suggested that the major factor in preferential hydration is the free energy required to form a cavity in the solvent, to accommodate the protein molecule (*7*). Because such a cavity would be larger for the unfolded protein, the free energy would increase and thereby the stability of the folded state is higher than the unfolded state (*8*). Polyols stabilize the protein by both steric repulsion and charge repulsion mechanism (*7*). The effect of various cosolvents on the activity and structural parameters of endoglucanase is important to understand the mechanism of its stabilization. Furthermore, these studies help in better utilization of these enzymes for various applications.

In the present paper, an effort is made to elucidate the effect of formulation variables (sorbitol, glycerol, and sucrose) on the

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structural stability of endoglucanase. This was investigated by activity measurements, fluorescence spectroscopy, thermal stability, and partial specific volume measurements. Thus, these data would ultimately provide the mechanism of protein−cosolvent interactions for better understanding at the molecular level, the forces that are responsible for the stability of the native molecule in these cosolvents.

MATERIALS AND METHODS

Materials. Crude cellulase from *Aspergillus aculeatus* was obtained from Novozymes (Bangalore). 2,4-Dinitrosalicylic acid (DNS), carboxymethyl cellulose (CMC), sucrose, glycerol, and sorbitol were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade, and quartz triple-distilled water was used in all of the experiments.

Purification of Endoglucanase. Endoglucanase was purified from crude cellulase of *A. aculeatus* by a salting out procedure using ammonium sulfate followed by ion exchange chromatography. The purity of the purified endoglucanase was evaluated by fast protein liquid chromatography (FPLC; Superdex-75 gel filtration column pre-equilibrated with 0.02 M sodium acetate buffer, pH 5.0, and eluted in the same buffer) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (PAGE) (12% gel). This was carried out according to the method described previously (*9*).

Assay of Endoglucanase. The reaction mixture consisted of 0.9 mL of 0.5% CMC in 0.02 M sodium acetate buffer, pH 5.0, and 0.1 mL of the enzyme solution. After incubation at 37 °C for 1 h, the reaction was stopped by the addition of 3 mL of DNS (1% alkaline solution of DNS) reagent. The solution was kept in boiling water bath for 5 min, and the volume was made up to 20 mL with water. The absorbance was measured at 520 nm (*10*) using a Shimadzu UV1601 spectrophotometer (Shimadzu, Asia-Pacific, Singapore). Glucose equivalents released were calculated from the suitable glucose standard curve. One unit of the enzyme activity corresponds to the release of 1 μmol equivalent of the reducing sugar (glucose) from substrate per minute. All of the enzyme activities were measured in the presence and absence of cosolvents in 0.02 M sodium acetate buffer after 2 h of preincubation. The activity of the endoglucanase in the absence of cosolvents served as a control, and the relative activity was calculated on the basis of its original activity. Stock solutions (50%) of cosolvents were prepared in the buffer, and the pH of the solution was adjusted to 5.0 (optimum pH) before making up the final volume. This was diluted to get different concentrations of cosolvents in which the enzyme solution was prepared and used for the assay.

Fluorescence Spectroscopy. Fluorescence emission spectral measurements were done using Shimadzu RF-5000 (Shimadzu Corp., Kyoto, Japan) automatic recording spectrofluorimeter. The temperature of the cell was maintained by a circulating water through the thermojacketed water bath. The excitation wavelength was fixed at 280 nm, and emission spectra were scanned from 300 to 400 nm (*11*) in buffer with and without cosolvent. The bandwidths for excitation and emission monochromators were fixed at 5 and 10 nm, respectively. The readings of relative fluorescence intensity or change in wavelength of maximum emission were plotted versus cosolvent concentrations. The fluorescence intensity of the protein in the absence of cosolvents (experimental conditions) served as a control, and the percentage relative fluorescence intensity was calculated on the basis of its original fluorescence intensity.

Thermal Stability Studies. Activity loss as a function of temperature was followed in sodium acetate buffer (0.02 M, pH 5.0) and in the presence of different cosolvents. The enzyme solution (0.5 mg/mL) was incubated for 1 h at different temperatures in the range of 20−90 °C. After the solution was cooled (keeping in ice), the residual activity was measured (37 °C) by transferring an aliquot to the assay mixture. The activity of the control (without incubation) enzyme was taken as 100% to calculate the residual activity.

To check the reversibility, the specific activity of the enzyme was measured after heating the enzyme at 90 °C for 1 h in the presence of the cosolvents and cooled in ice. The cosolvents were removed by gel filtration chromatography on Sephadex G-25 column pre-equilibrated with 0.02 M sodium acetate buffer of pH 5.0. The column was eluted in the same buffer, and the enzyme fractions eluted in the void volume was used for the reversibility studies. Proper blanks of enzyme solution alone eluted from the column were used for the assay.

Apparent Thermal Transition Temperature Studies. The effect of different concentrations of cosolvents on the thermal denaturation profile of endoglucanase was determined by using Cary100 Bio UV−visible spectrophotometer, M/S Varian Australia Pty Ltd. (Victoria, Australia). Measurements were done at different temperatures in the range of 20−90 °C with 1 °C/min increments in the temperature, and the spectrum was recorded at 287 nm. A protein concentration of 2.2×10^{-6} M was used for all of the experiments, and buffer with the respective cosolvent was used in the reference cell. The apparent thermal transition temperature (apparent T_m) was calculated by monitoring the progress of denaturation followed by changes in the absorbance at 287 nm as a function of temperature, which were converted to a van't Hoff plot to obtain a typical temperature-dependent isotherm (*12*, *13*). From the thermal denaturation profile, the fraction of protein in the unfolded state (F_u) was calculated using the standard equation (*14*).

$$F_u = (Y_n - Y)/(Y_n - Y_u) \quad (1)$$

where Y_n is the absorbance of the protein in the native state, Y_u is the absorbance of the protein in the unfolded state, and Y is the absorbance of the protein at different temperatures. The apparent thermal transition is defined as the temperature at which the value of F_u is 0.5. The results are averages of three experiments.

Circular Dichroic (CD) Spectra. CD spectra were obtained by using Jasco J-810 automatic recording spectropolarimeter (JASCO Corp., Tokyo, Japan) fitted with a 250 W xenon lamp. All samples were previously centrifuged and filtered through Millipore filters (0.45 μm pore diameter). Samples were analyzed in 0.1 cm optical path length cells in the far UV (200−260 nm) region in buffer with and without cosolvent. The molar ellipticity values were calculated using a mean residue weight of 115. The secondary structure of endoglucanase was analyzed using the computer program of Yang et al. (*15*). The reported CD values were the averages of at least three independent runs.

Partial Specific Volume Measurements. The partial specific volume of endoglucanase was measured using Anton Paar DMA 5000 density meter (Anton Paar, Graz, Austria) at 20.00 ± 0.005 °C according to the standard procedure. The densities of the solvent and the protein solution were measured, and the partial specific volume was calculated according to the standard procedure (*7*, *16*, *17*). The densities of the solvent and the protein solution were measured, and the partial specific volume was calculated using the equation

$$\phi = 1/\rho_0[1 - (\rho - \rho_0/C)] \quad (2)$$

where ρ and ρ_0 are densities (g/mL) of the solution and the solvent, respectively, C is the protein concentration (g/mL), and ϕ is the apparent partial specific volume. The value of ϕ was plotted as a function of protein concentrations. The value extrapolated to infinite dilution of the protein is the partial specific volume of the protein. The data were analyzed using three-component systems, namely, water, protein, and cosolvent as components 1, 2, and 3, respectively, according to the standard notation (*18*, *19*). The preferential interaction parameter for the three-component system was calculated according to the standard procedure (*16*, *17*). The preferential interaction parameter for the three-component system was calculated according to the equation

$$\xi_3 = (\delta g_3/\delta g_2)_{T,\mu 1,\mu 3} = \rho_0(\phi_2{}^0\text{-}\phi'_2{}^0)/1 - \rho_0\overline{\nu}_3 \quad (3)$$

where g_i is the concentration of the component i in g/g of water, μ is its chemical potential, T is the thermodynamic temperature, ρ_0 is the density of the solvent, $\phi_2{}^0$ and $\phi'_2{}^0$ are the partial specific volume of the protein at isomolal and isopotential conditions, and $\overline{\nu}_3$ is the partial specific volume of component 3. All of the measurements were an average of at least three independent experiments.

Statistical Analysis. The data analysis was carried out in triplicate. Means and standard deviations were computed using Microsoft Excel. Correlation coefficients were carried out to assess the changes in the residual activity after removal of the cosolvent and between the partial specific volume and the preferential hydration parameters (*20*).

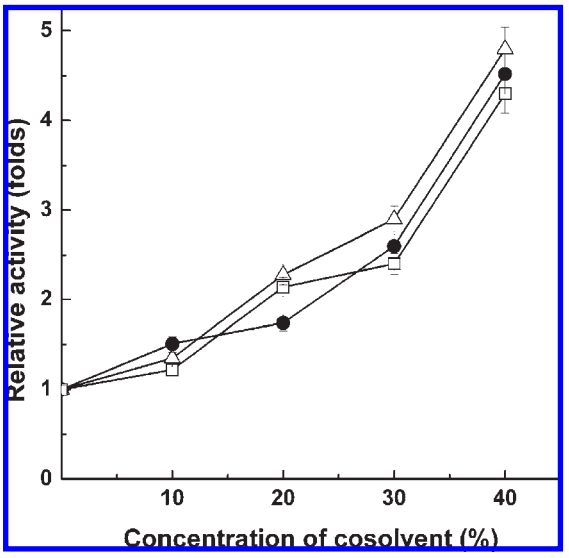

Figure 1. Relative activity of endoglucanase at its optimum temperature as a function of cosolvent concentration in 0.02 M sodium acetate buffer, pH 5.0. Sucrose (△), sorbitol (●), and glycerol (□).

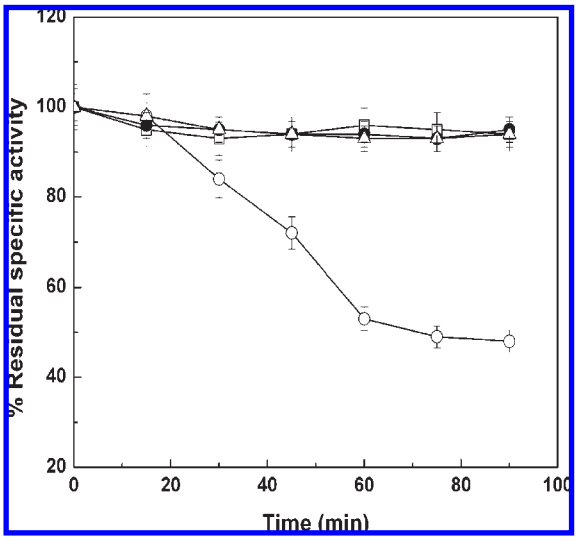

Figure 2. Effect of cosolvents on thermal inactivation of endoglucanase. The enzyme activity of samples heated at 90 °C with (40%) and without cosolvents was measured at pH 5.0 at 37 °C. Control (○), glycerol (□), sorbitol (●), and sucrose (△).

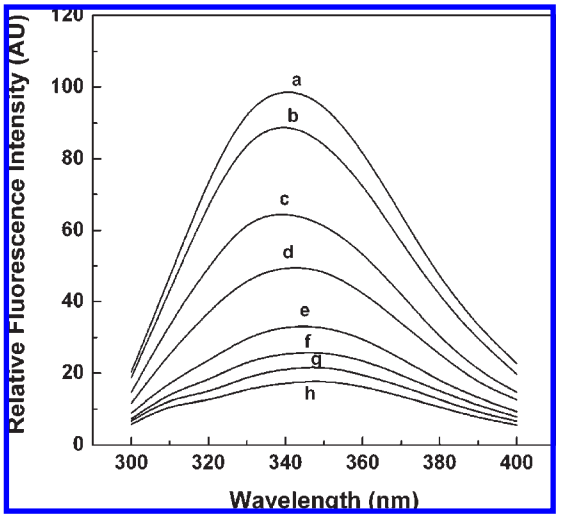

Figure 3. Fluorescence emission spectra in the wavelength range 300−400 nm as a function of temperature. The temperatures (in °C) used are (a) control (25), (b) 30, (c) 40, (d) 50, (e) 60, (f) 70, (g) 75, and (h) 80.

RESULTS

The analysis of the purified enzyme for its homogeneity by PAGE and FPLC showed a single band and a single peak, respectively, showing its purity as described previously (*9*). The effect of different concentrations of cosolvents (glycerol, sorbitol, and sucrose) on the activity of endoglucanase in 0.02 M sodium acetate buffer, pH 5.0, at its optimum conditions is shown in the **Figure 1**. With an increasing concentration of cosolvents, the activity of the enzyme increased by 4-fold at 40% concentration of the cosolvents after 2 h of preincubation.

The effect of cosolvents (sorbitol, sucrose, and glycerol) on the thermal stability of endoglucanase in buffered solution at pH 5.0 was measured through measurements of enzyme activity. Here, the enzyme with and without cosolvents was heated at 90 °C for different time intervals and then cooled to room temperature and the activity was measured with respect to time. From the data of activity versus time as shown in **Figure 2**, it was observed that the enzyme loses half of its activity at 90 °C in buffer alone, whereas in the presence of cosolvents, there was no loss in the activity. Therefore, the results show that all of the cosolvents have a good stabilizing effect and inhibit inactivation of endoglucanase at 90 °C. The fluorescence emission spectra (**Figure 3**) showed quenching of fluorescence intensity accompanied by red shift in the emission maximum above 60 °C. The enzyme showed 90% quenching with fluorescence emission maximum red-shifted to 351 nm at 80 °C. Therefore, the loss in enzyme activity may be due to changes in the conformation of the enzyme at higher temperatures. Stabilization by cosolvents was studied as a function of their increasing concentration. In all of the cases, the extent of stabilization was maximum at the highest concentrations of cosolvents (40%), where it retained almost 100% of its activity. Thus, from the enzyme activity measurements of endoglucanase in the presence of these cosolvents, it is clear that the endoglucanase molecule is stabilized against thermal inactivation.

To investigate the structural stabilization of endoglucanase as a function of cosolvents, fluorescence emission and far UV CD were monitored. **Figure 4A** shows the effect of increasing concentrations of cosolvents on the relative fluorescence intensity of endoglucanase. The increase in the intensity is gradual over the entire range of concentrations used. In addition to the increase in the fluorescence intensity, a 3 nm red shift in the wavelength of maximum emission was also observed at 30% concentrations of glycerol and sucrose except for sorbitol, where no change in the emission maximum was seen. The wavelength of maximum emission shifted from a control value of 336 nm to a value of 338 nm in the presence of 40% cosolvent. **Figure 4B** shows the change in the wavelength of maximum emission as a function of the concentration of these cosolvents.

The far UV circular dichroic spectra of endoglucanase (**Figure 5A**) showed a broad negative peak at 217 nm and a characteristic positive absorption band starting from ~207 nm of relatively weak magnitude (mean residue ellipticity −3400 deg cm^2/dmol), indicating the predominance of β-sheet structure (62% β-structures and 14% α-helix) in endoglucanase, which is consistent with other reported endoglucanases (*21*). Far UV CD spectra showed that the β-structure decreased with increasing temperature. At temperatures below 50 °C, there was no significant conformational change in the secondary structure of

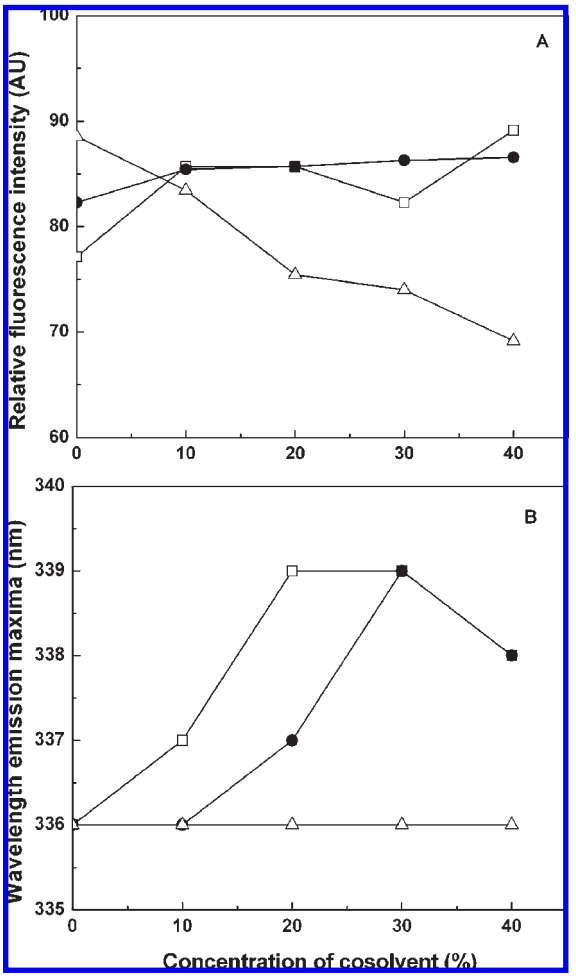

Figure 4. (**A**) Changes in the fluorescence intensity of endoglucanase as a function of cosolvent concentrations. (**B**) Changes in the fluorescence emission maxima of endoglucanase as a function of cosolvent concentrations. Glycerol (□), sorbitol (●), and sucrose (△).

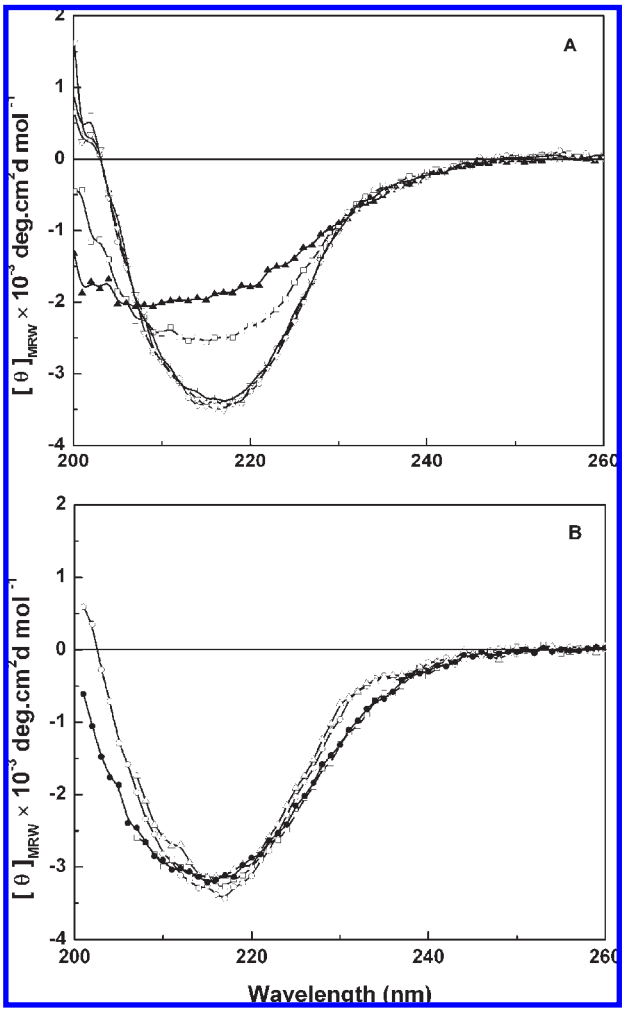

Figure 5. (**A**) Far UV circular dichroic spectra of endoglucanase in the wavelength range 200−260 nm at the following temperatures (in °C; without cooling): control (20) (○), 40 (▽), 50 (+), 55 (□), and 70 (▲). (**B**) Far UV CD spectra of endoglucanase in the presence and absence of cosolvents at 70 °C in 0.02 M sodium acetate buffer, pH 5.0. Native enzyme at 20 °C (○), enzyme exposed to 70 °C in the presence of 40% glycerol (□), enzyme exposed to 70 °C in the presence of 40% sucrose (△), and enzyme exposed to 70 °C in the presence of 40% sorbitol (●).

endoglucanase. At 70 °C, the β-sheet was found to be 54%, α-helix was 6%, and the random coil was about 34%. Above 50 °C, conformational changes occur in secondary structural levels, resulting in loss of the activity by 50% as shown in **Figure 2**.

The control sample (in the absence of cosolvents) had 50% of its residual activity after exposure to 90 °C for 1 h (**Figure 2**). In the presence of cosolvents, the original activity was almost retained (after exposure at 90 °C). **Table 1** shows the stabilization of the enzyme as indicated by the increase in activity with an increase in cosolvent concentration. It is clear that sucrose and glycerol at a 40% concentration offer maximum protection against thermal inactivation as observed after removal of cosolvent by gel filtration and had the specific activity values of nearly 76 and 66%, respectively. Sorbitol was the least effective of all of the cosolvents used, having a recovery value of 62% of its original activity. The stabilizing effect was maximum at 40% concentration for all of the cosolvents. The statistical analysis shows that the correlation coefficient is highly significant. In the presence of cosolvents, no significant changes were observed in the secondary structure of the protein at higher temperature as shown in **Figure 5B**. This clearly shows that the cosolvents protect the protein molecule against the thermally induced structural changes.

The stabilization of endoglucanase as shown by activity measurements has been further probed by the measurement of apparent thermal transition temperature (T_m) of endoglucanase both in the presence and in the absence of these cosolvents. Here, the changes in the absorbance of protein at 287 nm were monitored as a function of temperature in the range of 25−90 °C. **Figure 6** shows the thermal denaturation profile of endoglucanase in the presence of different concentrations of cosolvents. The thermal denaturation curve analysis of endoglucanase indicated a shift in the apparent thermal denaturation temperature as a function of different cosolvent concentrations. The apparent T_m increased from a control value of 57 ± 1 °C to a value of 66 ± 1 °C in presence of 40% sucrose. The apparent transition temperature value increased with an increase in the cosolvent concentrations. The apparent T_m of endoglucanase in the presence of 10, 20, and 30% of sucrose was 59, 61, and 64 ± 1 °C, respectively. Similarly, the increased thermal stability of endoglucanase was also seen in the presence of other cosolvents shown in **Figure 6**.

The partial specific volume of endoglucanase in the presence of different cosolvents like sucrose, sorbitol, and glycerol has been determined to understand the preferential interaction of endoglucanase with these cosolvents (**Table 2**). The preferential interaction parameters were calculated from the isomolal and

Table 1. Reversibility of Specific Activity[a] of Endoglucanase as a Function of Cosolvent Concentration after Exposure to 90 °C for 60 Min in Presence and Absence of Cosolvents

		residual specific activity (%)	
cosolvent	concentration (%) (w/v)	in the presence of cosolvent	after removal of the cosolvent by gel filtration
sorbitol	10	55 ± 2	51 ± 1
	20	62 ± 2	54 ± 1
	30	75 ± 2	60 ± 2
	40	92 ± 3	62 ± 2
glycerol	10	63 ± 2	51 ± 2
	20	70 ± 2	58 ± 2
	30	86 ± 2	62 ± 2
	40	97 ± 3	66 ± 2
sucrose	10	69 ± 2	57 ± 1
	20	119 ± 2	60 ± 2
	30	132 ± 3	68 ± 3
	40	145 ± 3	76 ± 3

[a] The residual specific activity of endoglucanase in 0.02 M sodium acetate buffer, pH 5.0, is 50 ± 2%. The cosolvent concentration shown is in the same buffer only. The correlation coefficient values for sorbitol, glycerol, and sucrose were found to be 0.94, 0.93, and 0.83, respectively. It was observed that the correlation coefficients were highly significant ($p < 0.001$).

isopotential partial specific volume measurements. The partial specific volume in the presence of cosolvents like sucrose, sorbitol, and glycerol indicated that endoglucanase is preferentially hydrated to different extents in all of the cosolvents used. The isomolal value of endoglucanase is in the range of 0.723 ± 0.001 mL/g for all of the systems. The isopotential value was highest in the case of 40% sucrose (0.756 ± 0.003 mL/g), and the value was lowest in the presence of 10% glycerol (0.731 ± 0.001 mL/g). **Figure 7** shows the change in the preferential interaction parameter ξ_3 in the presence of different concentrations of cosolvents. It is evident from **Figure 7** that the preferential interaction parameter increases with an increase in the concentration of the cosolvent for all of the cosolvents used. **Table 2** shows the partial specific volume, solvent composition, preferential interaction parameters, and related interaction parameters of endoglucanase in different cosolvents. The negative values of preferential interaction parameters indicate preferential exclusion of the solvent components from the domain of the protein molecules. The results indicate that the endoglucanase is stabilized in all of the cosolvents used in a concentration-dependent manner, and the preferential interaction parameter shows the extent of cosolvent exclusion. The maximum hydration was seen in glycerol (40%) where the preferential interaction parameter was −0.126 g/g and minimum value of −0.033 g/g in 10% glycerol. From **Table 2**, it is clear that the value of preferential interaction parameter on mol/mol basis is highest in 40% glycerol with a value of −61.90, while it was lowest in case of 10% sucrose with a value of −6.72 mol/mol. These results are highly and positively correlated, indicating the increasing trend with increasing concentrations of cosolvents.

DISCUSSION

The activity and structural stability measurements indicate the increased stability of endoglucanase in presence of cosolvents. Both the activity measurements of the enzyme at higher temperature and the measurements of apparent thermal denaturation temperature for endoglucanase showed that all of the cosolvents (sucrose, sorbitol, and glycerol) increased the thermal stability in a concentration-dependent manner in comparison with buffer alone. All cosolvents used in this study stabilize the

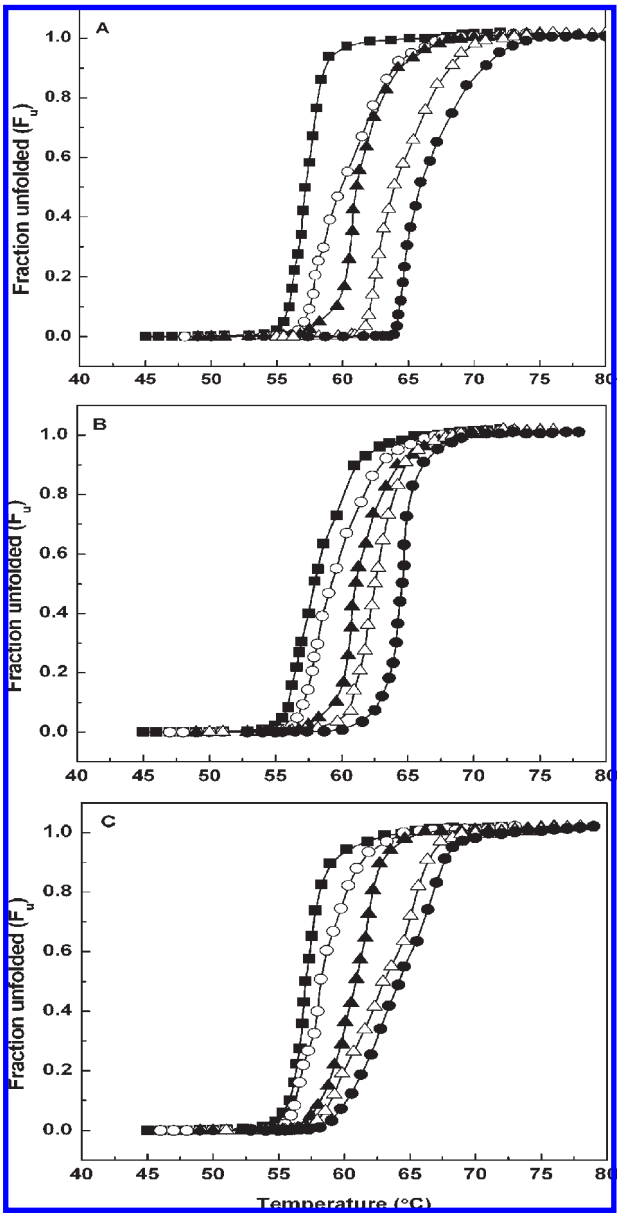

Figure 6. Thermal denaturation profile of endoglucanase in the presence and absence of cosolvents in 0.02 M sodium acetate buffer, pH 5.0, as a function of temperature in the range of 25−90 °C with a 1 °C/min increase in the temperature. The spectra were measured at 287 nm. In the presence of (**A**) sucrose, (**B**) sorbitol, and (**C**) glycerol. Control (■), 10% (○), 20% (▲), 30% (△), and 40% (●).

enzyme against thermal denaturation. The mechanism by which individual cosolvents bring about the stability may be different depending on the nature of the cosolvent used. Cosolvents stabilize proteins by various interactions like solvophobic, hydrogen bonding between sugars and proteins, an increase in surface tension, and preferential interactions (*22*).

The fluorescence studies of endoglucanase in the presence of these cosolvents showed changes in the microenvironment of the aromatic chromophore of endoglucanase to different extents in different cosolvents in a concentration-dependent manner. The fluorescence of tryptophan and tyrosine residues is sensitive to their microenvironment (*23*−*25*). Thus, fluorescence studies clearly show the difference in the mechanism of stabilization by individual cosolvents. The trypophanyl residues are more accessible to the solvent depending on the size of the solvent molecule. Glycerol has a higher accessibility being smaller than sorbitol and

Table 2. Preferential Interaction Parameters of Endoglucanase with Cosolvents

solvent	conc % (w/v)	ϕ_2^0 (mL/g)	$\phi'_2{}^0$ (mL/g)[a]	g_3 (g/g)	m_3 (mol of sol/1000 g water)	$(\delta g_3/\delta g_2)$ (g/g)	$(\delta g_1/\delta g_2)$ (g/g)	$(\delta m_3/\delta m_2)^b$ (*mol/mol*)
control (in buffer)		0.723 ± 0.001	0.723 ± 0.001					
glycerol	10	0.723 ± 0.001	0.731 ± 0.001	0.108	1.18	−0.033 ± 0.01	0.313 ± 0.04	−16.31 ± 1.40
	20	0.724 ± 0.001	0.735 ± 0.002	0.237	2.57	−0.051 ± 0.02	0.219 ± 0.03	−25.41 ± 4.40
	30	0.724 ± 0.001	0.743 ± 0.003	0.391	4.25	−0.099 ± 0.03	0.253 ± 0.03	−48.45 ± 6.40
	40	0.723 ± 0.001	0.745 ± 0.003	0.580	6.30	−0.126 ± 0.06	0.218 ± 0.03	−61.90 ± 8.40
sucrose	10	0.725 ± 0.001	0.744 ± 0.002	0.106	0.31	−0.051 ± 0.01	0.478 ± 0.02	−6.72 ± 0.90
	20	0.723 ± 0.001	0.746 ± 0.002	0.229	0.67	−0.068 ± 0.02	0.298 ± 0.03	−9.01 ± 1.20
	30	0.724 ± 0.001	0.751 ± 0.003	0.370	1.08	−0.079 ± 0.02	0.214 ± 0.03	−10.43 ± 1.40
	40	0.722 ± 0.001	0.756 ± 0.003	0.537	1.57	−0.109 ± 0.04	0.204 ± 0.03	−14.44 ± 2.10
sorbitol	10	0.723 ± 0.001	0.742 ± 0.002	0.107	0.59	−0.057 ± 0.01	0.538 ± 0.02	−14.35 ± 1.50
	20	0.723 ± 0.001	0.745 ± 0.003	0.231	1.27	−0.072 ± 0.02	0.313 ± 0.04	−18.02 ± 2.10
	30	0.726 ± 0.001	0.748 ± 0.002	0.375	2.07	−0.074 ± 0.02	0.197 ± 0.03	−18.40 ± 2.40
	40	0.723 ± 0.001	0.751 ± 0.003	0.548	3.03	−0.104 ± 0.03	0.190 ± 0.03	−26.01 ± 3.40

[a,b] The correlation coefficient values for partial specific volume (isopotential) and preferential interaction parameter on a mol to mol basis for glycerol, sucrose, and sorbitol were found to be 0.88, 0.88, and 0.70, respectively. The correlation coefficients were highly significant ($p < 0.01$).

sucrose (*26*). The presence of glycerol and sucrose might have altered the conformational changes in the protein molecule in the aromatic region, resulting in a shift in the emission maximum. Therefore, the changes in the fluorescence intensity cannot be considered as a major parameter in defining the solvent-mediated preferential hydration of the protein. In the presence of sorbitol, the preferential hydration and conformational changes might have taken place in the protein molecule in the region where the aromatic chromophores are absent. Thus, the results of fluorescence spectra indicate that no major conformational changes occur in the protein molecule.

The difference in the mechanism of stabilization of protein could be due to the polarity of the molecule. Nonpolar polyols bind to the hydrophobic sites of the protein, while the polar polyols to the hydrophilic sites. A study on protein conformation by Raman methods has shown that glycerol and sorbitol have little effect on the structural organization of water, suggesting that their protective effects arise from direct interaction with the protein molecule (*27*). Sugars enter the lattice structure of the water surrounding the protein molecule and strengthen it, thereby stabilizing the protein structure. The presence of sugars (*28*, *29*) and polyhydric alcohols (*30*) are known to stabilize the proteins to different extents depending on their nature. Trehalose is a better stabilizer as compared to other sugars and polyols due to its effect on the structure and properties of water (*31*−*33*). Timasheff and co-workers (*5*−*8*) and Prakash and co-workers (*26*, *34*, *35*) have shown the stabilizing effect of various cosolvents by preferential hydration in different proteins.

The effect of these cosolvents would be to shift the equilibrium to a less denatured state due to increase in the surface tension of water. This leads to preferential interaction of endoglucanase with different concentrations of cosolvents. This could bring about stabilization at higher temperatures as a result of preferential hydration in these solutions (*34*). The mechanism of stabilization according to Timasheff (*36*) may be explained in terms of preferential hydration. The cosolvent being bulkier molecules cannot enter the hydration layer present around the protein molecule and break the strong forces of attraction between the water and the protein surface. Therefore, they do not reach the protein surface as compared to water (*37*), and this phenomenon is known as preferential hydration. Our results have shown maximum preferential hydration at a higher (40%) concentration of cosolvents as compared to lower concentrations (10%). The preferential exclusion of the cosolvent molecules from the protein surface results in enhanced stability and activity of endoglucanase. The hydrophobic interaction could be

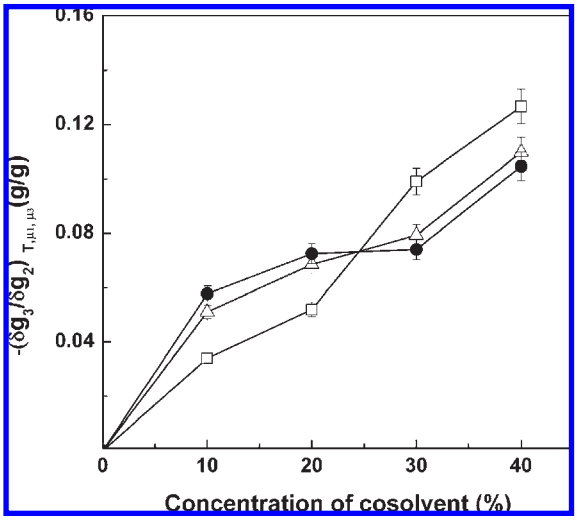

Figure 7. Preferential interaction parameter (ξ_3) of endoglucanase as a function of cosolvent concentrations in sodium acetate buffer, pH 5.0. Glycerol (□), sucrose (△), and sorbitol (●).

strengthened due to ordering of the water−cosolvent mixture around the enzyme. In the presence of cosolvents, there is reduction in hydrogen bonding between water molecules due to water−cosolvent interactions. This change in the water−water interaction favors the hydrophobic interactions between the nonpolar groups of the protein. This may be due to an increase in the surface free energy or surface tension of water, due to removal of cosolvent molecules at the interface, leading to preferential hydration (*28*, *38*, *39*).

The thermal transition temperature measurements clearly show that the apparent T_m of the enzyme increases with increasing concentrations of the cosolvents. Because the chemical potential of the protein is increased in the presence of cosolvents, it leads to a thermodynamically unfavorable condition (*40*−*42*). During the thermal denaturation, the enzyme unfolds, exposing the hydrophobic clusters of the protein to the solvent and increasing the surface area of contact between the protein and the solvent. In the presence of cosolvents, the unfolding of the protein molecule needs more energy than the aqueous solution alone, and such a reaction is thermodynamically unfavorable. Hence, higher temperatures are needed for the denaturation in the presence of these cosolvent systems. Thus, the presence of cosolvents favors a folded state of the protein molecule at higher temperatures. Preferential exclusion of cosolvent molecules from

the domain of protein molecule is the major driving force for the conformational stabilization through solvation effect. Low hydrophobicity of cosolvents ensures that the solvation shell around the exposed nonpolar groups of proteins remains intact at higher temperatures, leading to protein stabilization.

From the studies, it is clear that there is a considerable increase in the thermal stability of endoglucanase in the presence of cosolvents. It is evident that they protect or stabilize the protein by protecting its native structure by a preferential hydration mechanism. Thus, the structuring of water in the presence of cosolvents appears to be the dominant factor that governs such a stabilization process.

ABBREVATIONS USED

CMC, carboxymethyl cellulose; DNS, 2,4-dinitrosalicylic acid; T_m, apparent thermal transition temperature; CD, circular dichroism.

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