Characterization of Xylanase in DMSO-Water Mixtures
4.1 Introduction

Organic and mixed solvents are used increasingly in biochemistry and biotechnology to modulate the catalytic properties of enzymes and the stability of proteins and other biomolecules (Klibanov, 1989; Klibanov, 2001; Zaks and Klibanov, 1988a, 1988b; Zaks and Russell, 1988). However, not all organic solvents are equivalent and properties such as solvent hydrophobicity, hydrogen-bonding capacity and miscibility in water have profound effect on the structural integrity and catalytic activity of the enzymes. Protein in water immiscible hydrophobic solvents retain their native structure as a result of kinetic trapping which results from stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water (Mattos and Ringe, 2001). Conversely polar solvents (such as DMSO, DMF, formamide) easily strip off water from the surface of the protein and compete with water for protein-solvent hydrogen bonds. Polar solvents usually denature the structure and convert it to a largely unfolded state (Knubovets, et al., 1999). The conformation that a protein attains in solvent depends upon the ratio of the hydrophobic and hydrophilic areas on its surface. Changes in this ratio cause a rearrangement of the hydrogen bonds that result in conformational changes of the whole molecule (Avbelj and Moult, 1995; Carra and Privalov, 1996). Since the early studies by Singer's (1962) on protein behavior in non-aqueous media it is generally assumed that proteins are insoluble in most organic solvents with few exceptions such as dimethyl sulfoxide (DMSO), ethylene glycol, formamide and some halogenated alcohols (Jackson and Mantsch, 1992). DMSO is long known since nineteenth century as a by product of the wood industry and has aroused so large interest (particularly the DMSO-water mixtures) in the fast few decades that it has been claimed that "only rarely can a single compound (i.e DMSO) have found such manifold applications in so many fields" (Catalan, et al., 2001). It is perhaps the most extensively used aprotic (co)solvent in chemistry, biology, and medicine. DMSO is completely miscible with water in all proportions and like most organic liquids it is an amphipathic molecule with a highly polar domain and two apolar methyl groups. DMSO easily penetrates and diffuses through biological membrane and tissues.
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and protects bacterial and mammalian cells from damage during freezing and freeze storage (Lovelock and Bishop, 1959). Co-solvents that can stabilize a particular state of protein in one condition but destabilize under other conditions are combined co-solvents and DMSO is one of the prime solvents belonging to this class of molecules that has been extensively studied. The effects of DMSO on protein are quite variable and it can act as a stabilizer (Rajeshwara and Prakash, 1994; Rajendran, et al., 1995), denaturant (Bettelheim and senatore, 1964; Bhattacharjya and Balram, 1997; Fujita, et al., 1982; Hamaguchi, 1964; Jackson and Mantsch, 1991; Jacobson and Turner, 1980; Kotik, et al., 1995; Kovrigin and Potekhin, 1997), inhibitor (Kleinfeld, et al., 2000; Johannesson, et al., 1997; Perlman and wolff, 1968), as well as an activator (Amitabh, et al., 2002; Almarsson and Klibanov, 1996; Ramirez-silva, et al., 2001; 2003; Wolff et al., 1999), a chaperone (Kotik, et al., 1995; Ou, et al., 2002a), a cryoprotectant (Bragger, et al., 2000; Lovelock and Bishop, 1959, Yu and Quinn, 1994); a stabilizer of folding intermediate (Bhattacharjya and Balram, 1997), a modifier of protein conformation (Freire, et al., 1997; Villas-Boas, et al., 2002) and as a medium which allows wide variety of synthetic reactions which normally do not occur in water (Ferrer, et al., 1999; Pedersen, et al., 2002). Other than carrying out modulation of enzyme properties, DMSO also have pharmacological applications which have been discussed in detail by Santos et al., (2003).

4.1.1 DMSO-Water Interaction

Dimethyl sulfoxide (DMSO) is a powerful dipolar, very weakly acidic and fairly basic aprotic solvent with a dielectric constant of 46.45 and dipole moment of 4.0. Water also is highly polar, but in contrast to DMSO it is highly acidic and negligibly basic (Catalan, et al., 2001). As a result the two solvents interact strongly and their interaction is much stronger than of two water or two DMSO molecules respectively (Bell, et al., 1997). The greater association in DMSO-water mixtures can be explained in terms of the polar sulfoxide group. The DMSO molecule fluctuates between canonical forms I and II, in which the negatively charged oxygen atoms forms a hydrogen bond with water molecules.
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Fig. 4.1: Ionization of DMSO in DMSO-water mixture.

DMSO-water mixtures are characterized by their non-ideality. The deviation from ideality in viscosity, refractive index, density and enthalpy of aqueous DMSO solutions has been attributed to association interaction between water and DMSO (Aminabhavi and Gopalakrishna, 1995; Catalan, et al., 2001; Markarian, et al., 2007). The maximum deviation occurs at 30-40 mol % of DMSO and DMSO-water molar ratio of 1:2 (Luzar and Chandler, 1993). The average angle between two hydrogen bonds in water and DMSO in 1DMSO: 2H₂O complexes is nearly tetrahedral. However since DMSO can only accept and not donate H-bonds it’s presence in aqueous solution prevents the formation of large H-bonded networks of water molecules.

The variety of effects that DMSO displays on proteins has been well studied. However, no general mechanism yet proposed, can explain these results. Arakawa et al., (2007) have reported that at low concentrations, DMSO is preferentially excluded from the protein surface with significant binding occurring only at higher concentrations. Binding at higher concentrations is expected because of hydrophobic interactions between the two methyl groups and the non-polar groups on protein surfaces. DMSO therefore would play two different roles in aqueous protein solution:

1. Binding to the non-polar surface regions of the protein, as well as to hydrogen bond donors on the protein surface and
2. Association with water in the bulk solvent through hydrogen bonding

The observed exclusion from the protein surface at low DMSO concentrations is attributed to the large excess of water which would induce strong hydration of DMSO and thus increase the distance of hydrated DMSO molecule to the protein surface (Kita, et al., 1994).

4.2.2 DMSO and Protein behavior

In order to understand how DMSO influences the behavior of proteins, it is important to take into account the physical properties of DMSO-water mixture and the interactions of the protein with the solvent. The changes in the preferential interaction coefficient upon heat denaturation of Hen egg white lysozyme in aqueous solution of six various solvents: DMSO, methanol, ethanol, propanol, acetone, and p-dioxane have showed an anomaly with respect to DMSO and other solvents used (Kovrigin and Potekhin, 1997). The preferential interaction (solvation) changes (i.e the number of cosolvent molecules entering or leaving the solvation shell of protein upon denaturation) for homologous alcohols, acetone and dioxane do not depends on the nature of the solvent and is similar in the initial activity range. The similarity of preferential interaction coefficient values indicates that when one molecule of lysozyme denatures, the protein solvent shell included the same number of co-solvent molecule and lysozyme tends not to distinguish between these agents in the low activity region and preferential solvation has a nonspecific character. Although the general physical properties of DMSO are quite similar to the other five solvents, DMSO does not show similar changes in preferential interaction coefficient and the changes are much sharp and distinct to those as observed for the other five solvents used in the study. Recently the interaction of DMSO with proteins was reported using differential refractometry and amino acid solubility measurements (Arakawa, et al., 2007). The test proteins used in that study show negative preferential DMSO binding or preferential hydration at low DMSO concentration. However at higher concentrations of DMSO the interaction changed from preferential hydration to preferential binding (except for ribonuclease). The observed preferential
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hydration of the native protein has been correlated with excluded volume effects of DMSO and the unfavorable interaction of DMSO with polar component of the protein surface. DMSO also has been reported to reduce the thermal stability of monomeric globular proteins (RNase, lysozyme) without changing the unfolding mechanism (Fujita, et al., 1982; Jacobson and Turner, 1980; Kotik, et al., 1995; Kovrigin and Potekhin, 1997). It has been proposed that effect may be due to either or both the preferential solvation of the denatured state by DMSO (Kovrigin and Potekhin, 1997) and changes in the water structure (Fujita, et al., 1982). The effect of DMSO on the primary hydration shell and enzyme dynamics has been studied by MD simulations and it has been shown that DMSO strips off water molecules from the protein surface (Zheng and Ornstein, 1996a). In comparison to the proteins simulated under aqueous conditions DMSO appears to reduce the mobility of proteins and promotes electrostatic interactions and hydrogen bonds (Roccatano, et al., 2005; Zheng and Ornstein, 1996a).

While most proteins are soluble in neat DMSO (Chin, et al., 1994), their tertiary and even secondary structures are usually highly disrupted at high DMSO concentrations (Jackson and Mantsch, 1991). The denaturation of proteins by DMSO, as well as by other organic co-solvents, occurs in a sharp transition at a protein-dependent threshold concentration (Khmelmitsky, et al., 1991). In the case of lysozyme, for example, the tertiary fold is disrupted at a DMSO concentration around 70% (ca. 10 M) (Bhattacharjya and Balram, 1997; Fujita, et al., 1982). At lower DMSO concentrations minor structural perturbations and DMSO binding to the active site cleft and elsewhere on the protein surface have been observed spectroscopically.

One of the most remarkable behavior of proteins is specific recognitions of ligands, which is controlled by their active site topology and energy required to transfer the ligand to the binding site. It is seen that addition of DMSO can often perturb protein-ligand recognition phenomena. For example, in F1-ATPase, 30-40% of the DMSO increased the partitioning of the inorganic phosphate between the hydrophobic catalytic site and medium with low water activity (Tuena, et al.,
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1993). In phosphatidylinositol specific phospholipase C, the affinity for hydrophilic substrate increases progressively in 30 % isopropanol, 40 % DMF and 50 % DMSO. This has been correlated to increasing strength of H-bond between water and co-solvent which leads to increased substrate affinity. The partial dehydration of the hydrophobic active site in phospholipase C promotes better binding of the hydrophilic substrate to enzyme (Wu, et al., 1997).

Xylanases are enzymes which catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan. The substrate (xylan) is highly hydroxylated and engaged in multiple hydrogen bonding interactions (Wicki, et al., 2007a). Most of the natural isolates show optimum catalytic activity at temperature close to or above 50 °C. This is correlated with the opening of the thumb loop of the enzyme to accommodate the substrate at the active site (Muilu, et al., 1998; Murakami, et al., 2005). Inspite of being optimally active at high temperature an irreversible loss in activity is observed at temperatures close to or above the optimum temperature for substrate hydrolysis (Davoodi, et al., 1998). Stabilization of xylanases by changes in the surrounding solvent media (Breccia, et al., 1998; Cobos, et al., 2003; George, et al., 2001) and other enzymes is well documented. The stabilizations in presence of co-solvents are mainly brought about by changes in the surface tension or preferential interactions of the enzyme with the water which in turn affects the hydration layer of the proteins. DMSO as long been used as a cryoprotectant at low temperatures (Bragger, et al., 2000; Lovelock and Bishop, 1959; Yu and Quinn, 1994) but the reports on the mechanism underlying the stabilization is sparse and still not very clear. The present work focuses on the effect DMSO on the activity and stability of xylanase in DMSO-water mixtures.
4.2 Materials and Methods:

4.2.1 Activity Profile in Polar Aprotic (DMSO, DMF, and MeCN) Solvents:

Enzyme activity was measured as a function of varying concentration of aprotic solvents in water. All activity measurements were carried out using the DNS assay method (section 3.2.20) at pH 7.0 in 50 mM phosphate buffer and 60 °C. Residual activity (defined as the activity in presence of added polar aprotic component w.r.t to that in its absence i.e., 0 % organic solvent in reaction mixture) was plotted against increasing concentration of the added solvent to get the activity curve as a function of the added polar-aprotic component.

4.2.2 pH and Temperature Activity Profiles of Xylanase

The pH activity profiles of native xylanase were obtained by measuring the enzyme activity at pH values from 3.0-9.0 in a gradient of 0.5 in Britton-Robinson buffer solutions (Palackal, et al., 2003) at 60 °C. Enzyme activity was monitored by measuring equivalents of reducing sugar as determined by the DNS assay method as described earlier (section 3.2.20). Relative activity profile as a function of pH was plotted to obtain the pH optima of the enzyme.

For temperature activity profiles, activity was monitored at a temperature range of 25 °C to 70 °C at pH 7.0 in 50 mM phosphate buffer. Relative rate of hydrolysis per minute was plotted as a function of temperature to obtain the temperature optima of the enzyme.

pH and temperature activity profiles in DMSO-water mixture were obtained in presence of DMSO at a concentration of 12 % (v,v) in the reaction mixture without any correction for the effect of added DMSO on the pH values.

All the activity measurements were done using oat spelt xylan as a substrate at a concentration of 10 mg/ml.
4.2.3 Calculation of Michaelis-Menten constant ($K_m$) & $V_{max}$

For calculating the Michaelis-Menten constants ($K_m$) and $V_{max}$ the enzyme activity was measured in varying substrate concentrations (2 mg/ml - 16 mg/ml). The double reciprocal plot for the initial rates of hydrolysis (in the linear region) against increasing substrate concentration was used to obtain the $K_m$ and $V_{max}$ value.

4.2.4 Spectral Measurements

4.2.4.1 Measurement of Stock Concentration

**Protein:** The concentration of the stock solution of protein was determined using a calculated extinction coefficient of 84340 M$^{-1}$ cm$^{-1}$, at 280 nm as described by Pace et al., (1995).

**NATA:** A stock solution of NATA (10 mM) in 20 mM phosphate buffer (pH 7.0) was prepared and concentration determined using an extinction coefficient of 5600 M$^{-1}$ cm$^{-1}$ at 280 nm.

**ANS:** A 20 mM stock of ANS was prepared in methanol and the actual ANS concentration was determined after dilution in water using the extinction coefficient of 5000 M$^{-1}$ cm$^{-1}$, at 350 nm.

a. ANS and NATA were obtained from Sigma (USA).

4.2.4.2 UV Absorption Spectroscopy:

Absorbance spectra of the xylanase in native and in DMSO-water mixtures were recorded on Cary varian spectrometer coupled with a peltier controlled water bath. Spectra were recorded in 1 cm pathlength quartz cuvettes at a protein concentration of about 4-5 µM. Absorbance spectra were recorded in a wavelength range of 250-320 nm at 25 ºC unless otherwise stated. The spectra were recorded with a scan speed of 100 nm/min with data interval set to 0.167 sec. The averaging time was 0.1 sec and the spectral bandwidth was set at 2.0.
Final spectra reported are an average of 3 scans after subtraction of the respective buffer baselines.

**4.2.4.3 Fluorescence Spectroscopy**

Fluorescence spectra were recorded on Cary eclipse spectofluorimeter in 1 cm pathlength quartz cuvette at a protein concentration of 1-2 μM. Spectral measurements were carried out at a scan speed of 1 nm/sec in and averaged over three scans. The excitation wavelength was set at 295 nm for selective tryptophan excitation and emission spectra were recorded between 300 to 450 nm. The excitation and emission slits were set at 5 nm each. Final Spectrum was obtained after subtraction of the buffer baselines to avoid any contributions from the Raman band of water (Creighton, 1997). All the spectra were recorded at 25 °C at a PMT voltage of 600 volts unless otherwise stated.

**4.2.4.4 CD Spectroscopy**

CD measurements were carried out using a Jasco-815 spectropolarimeter equipped with a peltier controlled temperature bath. In Near UV region the spectra was recorded between 250-320 nm at a protein concentration of 20 μM. The spectrum was recorded in a 1 cm path length quartz cell at 25 °C in continuous scanning mode at a scan speed of 5 nm/min. The data pitch and response time was set at 0.2 nm and 4 sec respectively with band width value set at 2 nm.

In the Far UV region the spectra were recorded between 200-260 nm with a protein concentration of 5 μM in 0.1 cm path length quartz cell. The spectral measurements were carried at 25 °C with a scan speed of 10 nm/min. The data pitch and response time was set at 0.5 nm and 1 sec respectively with band width value set at 1 nm.

The obtained ellipticity in millidegresses values were converted to molar ellipticity using the relation:

\[[\Theta] = \Theta \cdot M_r / 10cl\]
Where, $\Theta$ is the observed ellipticity in millidegrees, $M_r$ is the molecular weight, $c$ is the protein concentration (mg/ml) and $l$ is the path length (cm). All spectra reported are an average of 3 scans after the subtraction of respective buffer baselines.

### 4.2.5 Thermal Inactivation Measurements

The rate of inactivation of xylanase at higher temperatures was monitored using fluorescence measurements. Kinetic profiles were obtained on a Cary Eclipse spectofluorimeter equipped with a peltier controlled temperature bath. Excitation and emission wavelengths were set at 295 and 345 nm respectively. The excitation and emission slits each being set at 5 nm with the PMT detector voltage at 600 volts. The buffer was pre-warmed to the required temperature and was allowed to equilibrate in the thermal block for 5-10 min in the teflon stoppered quartz cuvette of path length 1 cm. Protein solution (pre-warmed to 25 °C) was then added to a concentration of 2 μM and the kinetics was monitored up to 60 min with an averaging time of 0.125 sec.

Irreversible thermal inactivation times in absence or presence of DMSO (12 % v/v) were also determined using activity measurements. For this an aliquot of the enzyme was mixed with substrate in a final reaction volume of 500 μl with or without DMSO (12 %, v/v) and was heated for varying time periods at 60 °C in temperature controlled water bath. The residual enzyme activity after cooling the reaction mixture to room temperature was determined using DNS assay method as described earlier in section 3.2.19 and 3.2.20.

### 4.2.6 Light Scattering Measurements

Light scattering measurements were carried out on Cary varian spectrometer coupled with a peltier controlled water bath. The buffer solution (20 mM phosphate, pH 7.0) was pre-warmed to 60 °C and was allowed to equilibrate with the thermal block for 10-15 mins in the teflon stoppered quartz cuvette of path length 1 cm. Protein solution (pre-warmed to 25 °C) was added to a
concentration of 5 μM and absorbance was recorded as a function of time at 360 nm. The averaging time was set at 0.1 sec with spectral bandwidth at 1.0.

### 4.2.7 Thermal Unfolding

Thermal unfolding studies were carried out on Cary varian spectrometer coupled with a peltier controlled water bath. Protein solution at a concentration of 5 μM was heated from 25 °C to 80 °C at a rate of 1 °C/min. Changes in the absorption were monitored at 293 nm at data intervals of 0.1 nm each. The averaging time was set at 0.5 sec with spectral bandwidth at 1.0. All the unfolding studies were carried out in 20 mM phosphate buffer (pH 7.0) in presence of 2.5 M urea. Any changes in pH due to the additions of DMSO were not taken into account.

### 4.2.8 Chemical Denaturation Measurements

Equilibrium unfolding studies of native xylanase were carried out at varying concentration of the denaturant (GdHCl or urea). In the presence of DMSO, only the GdHCl induced unfolding was monitored. Protein sample was equilibrated overnight with varying concentrations of the denaturant (in presence or absence of DMSO) in 20 mM phosphate buffer (pH 7.0) at 25 °C. The fluorescence spectrum was recorded as described earlier in the range of 300-450 nm after excitation at 295 nm. To consider both the variation in fluorescence intensity, and red shifting, the average emission wavelength ($\langle \lambda \rangle$) was used for data analysis. The average emission wavelength ($\langle \lambda \rangle$) was defined as,

$$\langle \lambda \rangle = \frac{\sum_{i}^{\lambda_e} (F_i \lambda_i)}{\sum_{i}^{\lambda_e} F_i}$$

Where, $F_i$ is the fluorescence intensity at emission wavelength and $\lambda_i$. Since average emission wavelength is an integral measurement, it has less error than

b. GdHCl and Urea was of highly pure grade ad obtained from Sigma (USA). DMSO was of analytical grade and obtained from Spectrochem (Mumbai).
measurements at a single wavelength. The average emission wavelength $\langle \lambda \rangle$ was plotted as a function of GdHCl concentration to get the denaturation curve and analyzed according to a two state model,

$$N \leftrightarrow U$$  \hspace{1cm} (i)

The pre and post transition baselines were extrapolated into transition region and the equilibrium constant $K$, and the free energy change, $\Delta G$ was calculated using the equation,

$$K = \frac{[(Y)_N - (Y)]}{[(Y) - (Y)_D]}$$  \hspace{1cm} (ii)

$$\Delta G = -RT \ln K = -RT \ln \frac{[(Y)_N - (Y)]}{[(Y) - (Y)_D]}$$  \hspace{1cm} (iii)

Where, $R$ is the gas constant (1.987 cal mol$^{-1}$ K$^{-1}$), $T$ is the absolute temperature, $(Y)_N$ and $(Y)_D$ are the measured signals of the native and unfolded states ($Y$) is the measured value at a particular denaturant concentration.

$\Delta G$ as a function of GdHCl concentration was plotted using eqn (iv) to obtain the values of $m$ and $\Delta G_d(H_2O)$

$$\Delta G = \Delta G_d(H_2O) - m[GdHCl]$$  \hspace{1cm} (iv)

$\Delta G_d(H_2O)$ is the free energy of unfolding in absence of any denaturant and $m$ gives a measure of conformational stability of the protein.

**4.2.9 ANS Binding Assay**

A fixed amount of protein solution (2 µM) was titrated with increasing concentrations of ANS up to 50-fold molar excess (100 µM of ANS) from a concentrated stock so that the volume fluctuations were minimal (less than 2%). The excitation and emission slits were set at 5 nm each. The fluorescence
spectrum was recorded as described earlier in the range of 400-600 nm after excitation at 370 nm.

4.2.10 *Fluorescence Lifetime Measurements*

Xylanase contains 11 tryptophans in various time-varying structural environments. This would lead to complex multi-exponential decay profiles of the fluorescent intensity. Traditional analysis of fluorescence life time data attempts to deconvolute the observed time-dependent decay of fluorescence intensity and extract the time-constants of every underlying component. In view of the large number of tryptophanyl residues in xylanase, such an approach was not thought to be feasible. We therefore used an alternative method of analysis in which the protein was assumed to be in a large number of conformations that can interconvert, at room temperature, at rates similar to the excited state decay rates.

Following this assumption the fluorescence intensity decay profiles can be modeled using a continuous distribution of lifetime values. The fluorescence intensity decay profile is now given by;

\[ I_f(t) = \sum_{i=1}^{m} A_i(\tau_i) e^{t/\tau_i} \]

Where, m is the number of independent decay processes, and \( A_i(\tau_i) \) is the fraction of light contributing to that component. It is further assumed that the total intensity of each component i.e \( A_i(\tau_i) \) depends on the number of tryptophans in that particular environment.

The fluorescence life time measurements of the protein and NATA in presence of varying concentrations of DMSO were performed on custom designed spectrofluorimeter in which time resolved (Model FL900) and steady state (Model FS900 both from Edinburgh Instruments, UK) has been integrated. The excitation source was a hydrogen gas filled flash lamp in which the gap distance between the two electrodes was set at 0.8 mm. The hydrogen gas pressure was 0.4 bars and
repetition frequency was set at 40 kHz. The data acquisition was based on TCSPC (time correlated single photon counting) and about 10,000 counts were acquired for each decay profile. The instrument response function (IRF) was recorded after each measurement using a scattering solution of barium sulphate.

The data were analyzed in the fitting range of 100 -700 channels using FAST software package (Edinburgh Instruments) with Life time distribution analysis subroutine. The f value (i.e. A_i(τ_i)) were obtained from the integrated area of each component that contribute to that intensity decay function.
4.3 RESULTS:

4.3.1 Activity Profile of Xylanase in Polar Aprotic (DMSO, DMF, and MeCN) Solvents:

In order to study the behavior of xylanase in the polar aprotic solvents, activity profiles were monitored in binary water-solvent mixtures consisting of either of the three solvents: DMSO, DMF and MeCN. In all the three solvent systems, loss in enzyme activity was observed with increasing concentrations of the added polar aprotic component. Fig. 4.2 shows the residual activity profile of xylanase as a function of the solvent concentration (v,v).

![Activity profile of purified recombinant xylanase as a function of polar aprotic organic solvents concentration. Residual activity is defined as the activity in presence of added polar aprotic component w.r.t to that in its absence (i.e 0 % organic solvent in reaction mixture). The points represent residual activity data obtained in duplicate experiments. The curves passing through the data points are simply to guide the eye.](image)

Fig 4.2: Activity profile of purified recombinant xylanase as a function of polar aprotic organic solvents concentration. Residual activity is defined as the activity in presence of added polar aprotic component w.r.t to that in its absence (i.e 0 % organic solvent in reaction mixture). The points represent residual activity data obtained in duplicate experiments. The curves passing through the data points are simply to guide the eye.
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In case of DMF and MeCN, the activity falls very rapidly and there is no detectable activity beyond 6 % (v,v) and 8 % (v,v) of the organic solvent respectively. However, the rate of decline in activity is not as sharp in the case of DMSO. At around 12 % (v,v) of the DMSO the enzyme activity is diminished to half of its value in absence of DMSO and no detectable activity was observed only at or beyond 30 % (v,v). This concentration of DMSO (i.e., 12 % v,v) lies in the sharp transition region of protection of xylanase against thermal induced aggregation (section 4.3.4) and was hence used in further studies (using activity measurements) on the behavior of xylanase in DMSO-water mixtures.

The $[C]_{1/2}$ values (i.e. the solvent concentration at which the activity is reduced to half of the original value) in the solvent systems along with relevant physical properties of the solvent are given in Table 4.1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$[C]_{1/2}$</th>
<th>Dipole moment($\mu$)</th>
<th>Dielectric Const.(\epsilon)</th>
<th>logP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>12 %</td>
<td>4.0</td>
<td>46.5</td>
<td>-1.13</td>
</tr>
<tr>
<td>MeCN</td>
<td>5%</td>
<td>3.4</td>
<td>35.9</td>
<td>-0.33</td>
</tr>
<tr>
<td>DMF</td>
<td>3%</td>
<td>3.9</td>
<td>36.7</td>
<td>-1.00</td>
</tr>
</tbody>
</table>

Table 4.1: Solvent Parameters for DMSO, MeCN and DMF and the $[C]_{1/2}$ values for substrate hydrolysis by xylanase in these solvent system.

c. Dipole moment ($\mu$) gives the measure polarity of a polar covalent bond and is defined as the product magnitude of charge on the atoms and the distance between the two bonded atoms.

d. Dielectric constant is defined as the ratio of the permittivity of a substance to the permittivity of free space and gives the measure for the effect of a medium on the potential energy of interaction between two charges.

e. logP gives the measure of hydrophobicity of the solvent and is defined as the logarithm of the partition coefficient of the solvent in a standard two-phase system of 1-octanol and water.
4.3.2 pH and Temperature Activity Profile of Xylanase in Absence and Presence (12 % v,v) DMSO:

pH and temperature activity profile of the xylanase was monitored in absence and presence (12 % v,v) DMSO i.e., at the $[C]_{1/2}$ value. The pH and temperature activity profiles are shown in Fig. 4.3 and 4.4 respectively. In both presence (12 % v,v) and absence of DMSO the pH activity profile showed a bell shaped curve. The slopes of the ascending and descending limbs of the bell shaped curves were similar. The pH optima of the enzyme in absence of DMSO was close to 6.5 while in presence of 12 % DMSO the pH optima was close to 5.5 (Fig 4.3).

![Relative activity profiles of purified recombinant xylanase as a function of pH in: A). 0 % DMSO, B). 12 % DMSO-water mixture. Relative activity is defined as the activity at a given pH w.r.t to the activity at optimum pH (i.e highest activity) in the respective solvent system at the given temperature. The points represent relative activity data obtained in triplicate experiments. The curves passing through the data points are simply to guide the eye.](image)

Fig 4.3: Relative activity profiles of purified recombinant xylanase as a function of pH in: A). 0 % DMSO, B). 12 % DMSO-water mixture. Relative activity is defined as the activity at a given pH w.r.t to the activity at optimum pH (i.e highest activity) in the respective solvent system at the given temperature. The points represent relative activity data obtained in triplicate experiments. The curves passing through the data points are simply to guide the eye.
The acidophilicity or alkalophilicity of the xylanases is controlled by the presence of Asp/Asn residue in near vicinity (position 35 in *Bacillus circulans*) of the catalytic glutamates (Glu 78 and Glu 172). The pH optimum is also dependent on the electrostatic interactions between residues at the active site which in turn controls the pKa of the catalytic glutamates and is controlled by an intricate network of H-bonds at the active site region (Joshi, *et al.*, 2000, Joshi, *et al.*, 2001). The pKα cycling (McIntosh, *et al.*, 1996) between the catalytic glutamates is necessary for the catalysis and can be perturbed by the addition of DMSO which in turn affects the optimum pH for substrate hydrolysis by the xylanase.

The temperature activity profile showed increase in rate of substrate hydrolysis with increasing temperatures with optimum temperature being 60 °C (Fig. 4.4) in absence of DMSO. However, beyond 60 °C the loss in activity was very sharp and an increase of 10 °C above the temperature optimum lead to a loss of ~70 % in the enzyme activity. The increase in rate of hydrolysis with increasing temperatures was also observed in 12 % (v,v) DMSO-water mixtures (Fig 4.4). The temperature optimum for substrate hydrolysis in DMSO-water mixture (12 % v,v DMSO) was 55 °C (at pH 6.5) with a loss of ~90 % of enzyme activity at 70 °C.

The temperature optimum profile of the xylanase in 0 % and 12 % (v,v) DMSO clearly shows that at low temperatures the relative activity in 12 % DMSO is higher than that of respective relative activity in 0 % DMSO indicating that at low temperatures DMSO might increase the turnover rate and hence in turn catalysis.
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Fig 4.4: Relative activity profiles of purified recombinant xylanase as a function of temperature in: A), 0 % DMSO, B). 12 % DMSO water mixture. Relative activity is defined as the activity at a given temperature w.r.t to the activity at optimum temperature (i.e highest activity) in the respective solvent system at the given pH. The points represent relative activity data obtained in triplicate experiments. The curves passing through the data points are simply to guide the eye.

With respect to the aqueous conditions (Fig 4.3 and 4.4) the pH and temperature dependency in DMSO-water mixture at 12 % v.v DMSO showed a shift of pH optimum by 1.0 units and temperature optimum by 5 °C. However, the observed values of pH and temperature optimum for the purified recombinant xylanase in native (i.e, in absence of DMSO) were in agreement with the reported value for the native xylanase produced by Bacillus coagulans (Chauhan, et al., 2006; Choudhury, et al., 2006).

4.3.3 Calculation of Michaelis-Menten constant (\(K_m\)) and \(V_{max}\):

Xylanase catalyzed hydrolysis rates for oat spelt xylan were determined in a range of substrate concentrations up to 16 mg/ml. In the aqueous condition (i.e. 0 % DMSO) the plot of hydrolysis vs. substrate concentration showed saturation beyond 12 mg/ml of substrate. In presence of 12 % DMSO (v,v) the initial rate of
hydrolysis was slower as compared to that in aqueous conditions and the saturation phase was not achieved till the measured substrate concentrations. Limited solubility of the oat spelt xylan precluded the activity measurements at higher substrate concentrations. The double reciprocal plot of the initial rates of hydrolysis is shown in Fig. 4.5. The enzyme kinetics data and the kinetic parameters are listed in Table 4.2.

![Double-reciprocal plot of the substrate hydrolysis by purified recombinant xylanase as a function of substrate (oat spelt xylan) concentration in absence and presence of (12 % v,v) DMSO.](image)

**Table 4.2: Kinetic parameters ($K_m$ and $V_{max}$) for substrate (oat spelt) hydrolysis by xylanase at 60 °C, pH 7.0 in 0 % and 12 % (v,v) DMSO**
The obtained $K_m$ value for the hydrolysis of oat spelt xylan by the recombinant xylanase was in good agreement with that of reported for family 11 xylanase (Nath and Rao, 2001a). However the $V_{\text{max}}$ values differed slightly which could be due to the difference in the method used for the estimation of reducing sugar equivalents released. One observes that the $K_m$ for xylanase increases 4 fold when DMSO is added at a concentration of 12 % (v,v). The $V_{\text{max}}$ also increases ~ 2.8 fold at 12 % (v,v) of DMSO. The increase in $K_m$ with change in solvent properties is clearly due to a structural change in enzyme that alters the binding site for the xylan substrate. Interestingly $V_{\text{max}}$ also increases. This may be due to a change in the ionization equilibrium of the catalytic glutamates (Glu 78 and Glu 172) brought about by the DMSO induced lowering of the dielectric constant of the medium.

4.3.4 Influence of DMSO on the Temperature Induced Inactivation of Xylanase

The contribution to the UV absorption spectrum of xylanase is solely due to the aromatic amino acids (4 Phe, 16 Tyr and 11 Trp). It is devoid of any cysteine. The UV absorbance spectrum of the xylanase recorded in the absorbance region of aromatic chromophores Phe, Trp and Tyr showed a shoulder in ~ 290 regions (Fig. 4.6 A & B) which is due to the unusually high number of tryptophans for a relatively small globular protein of the size of xylanase. The UV absorbance spectra of xylanase as a function of temperature showed no noticeable change up to 45 °C however, at 50 °C or above an increase in absorbance was observed but along with formation of formation of aggregates. The difference spectrum of the temperature dependent changes between 50 °C and 25 °C showed peaks at 286 and 293 nm (data not shown). Absorbance spectra recorded in varying concentration of DMSO (up to 30 % v,v) as a function of temperature also showed no noticeable change up to 40 °C as in the case of native i.e., 0 % of DMSO (data not shown).
Characterization of Xylanase in DMSO-Water Mixtures

The ultra violet absorbance spectrum as a function of DMSO concentration at two temperatures 25 °C & 40 °C is shown in Fig. 4.6 A & B. At both the temperatures increase in absorbance was observed with increasing DMSO concentrations. The concentration dependent difference spectrum shows sharp peaks around ~285 and ~291 nm which is the region for tryptophan absorbance. However, at 40 °C a clear reduction in the magnitude of the sharpness of peaks was observed (Fig 4.6 C & D).

**Fig. 4.6:** A & B. Ultraviolet absorbance spectra of purified recombinant xylanase as a function of DMSO at 25 °C and 40 °C. C & D. Difference spectra of purified recombinant xylanase between 0 % and X % DMSO as a function of DMSO concentration at 25 °C and 40 °C (where, X= DMSO concentration (v,v) in which the spectrum was recorded).
Characterization of Xylanase in DMSO-Water Mixtures

The difference in the sharpness of the peaks in difference spectra suggests that the perturbation by DMSO at higher temperatures is not similar to that at lower temperatures. A comparison of Fig. 4.6 C & D also clearly shows the disappearance of 291 nm peak and rise of a new shoulder at around 278 nm (at 40 °C with increasing DMSO concentration) indicating alteration of the protein structure leading to exposure of additional tyrosine residues that have characteristic absorption peak around 274 nm.

The effect of DMSO on protein conformation was further probed by fluorescence spectroscopy. The properties of the protein molecule in absence of any external chromophore depends on three aromatic acids Phe, Tyr, Trp. The quantum yields for the Phe is very low. Further the emission spectrum of Phe and Tyr overlaps with the absorption region of Tyr and Trp respectively. In a protein containing all these three fluorophores (such as xylanase) not much information can be extracted out using either Phe or Tyr as a probe. Hence all the fluorescent studies were done by selective excitation of tryptophan fluorophore (λ<sub>ex</sub> 295 nm).

The fluorescent spectra of xylanase in varying concentration of DMSO (Fig 4.7) at 25 °C showed slight increase in the fluorescent quantum yield with increasing DMSO concentrations. A peculiar observation was blue shift of fluorescent spectrum at higher DMSO concentrations. The λ<sub>max</sub> for the emission spectrum of xylanase in 0 % DMSO to 20 % DMSO changed from ~345 nm to ~337 nm (Fig 4.7). A similar change was observed for the Trp analogue NATA where the λ<sub>max</sub> for the emission changes from ~ 360 nm which gradually changes to ~ 355 nm as one goes from 0 % to 20 % (v,v) of DMSO (data not shown). The observed blue shift can be accounted by the changes in the polarity of the surroundings of the chromophore (i.e Trp residues) upon DMSO addition or due to sequestering of tryptophan residues in a more rigid environment due to the loss in hydration upon DMSO additions. No time dependent changes in the emission intensity of xylanase at 345 nm (λ<sub>ext</sub> 295 nm) were observed at 25 °C with increasing concentrations of DMSO up to 30 % (v,v) of DMSO (data not shown).
Characterization of Xylanase in DMSO-Water Mixtures

![Fluorescence spectra of xylanase in DMSO-water mixtures](image)

**Fig. 4.7:** Fluorescence spectra of purified recombinant xylanase as a function of DMSO concentration.

As shown in Fig. 4.8, the fluorescent spectrum of xylanase in absence of DMSO showed a gradual decline in the fluorescent intensity as function of temperature up to 50 °C. This is because of the fact that the tryptophan fluorescence itself undergoes quenching effects with increase in temperature (Creighton, 1997). However time dependent changes were observed at higher temperatures (close to or above 55 °C). After an incubation of ~10-15 min at 55 °C the fluorescence first increases sharply which thereafter progressively reduces at longer incubation periods (Fig 4.8A). The difference spectrum of the temperature dependent changes between 55 °C and 25 °C showed peaks at positions 345 nm (data not shown). Fig.4.8B shows the changes in relative fluorescence intensities at 40 °C and 55 °C. It is quite clear that the xylanase undergoes distinct changes at 55 °C but not at 40 °C. This indicates that at 55 °C or higher xylanase undergoes a slow irreversible deactivation process. This is consistent with the formation of aggregates as observed earlier from UV measurements.
**Characterization of Xylanase in DMSO-Water Mixtures**

*Fig 4.8: A*. Fluorescence spectra of purified recombinant xylanase as a function of temperature. *B*. Fluorescence emission kinetics at 40 °C and 55 °C (λ<sub>ex</sub> 295 nm and λ<sub>em</sub> 345 nm).

Since the temperature dependent inactivation of xylanase appeared to be due to the formation of insoluble aggregates, we decided to use light scattering technique as a direct measure of the aggregation process. The light scattering measurements were carried out as a function of DMSO concentration at 60 °C. The time dependent increase of absorbance values at 360 nm showed a gradual loss in the behavior of aggregate formation with increase in DMSO concentration (Fig 4.9A). A sharp transition was observed at 10-15 % (v,v) DMSO and no aggregate could be detected at a DMSO concentration of 15 % (v/v) or above using UV light scattering measurements (Fig 4.9B).

Similar results were also obtained with increasing amounts of the commonly used urea and GdHCl. In urea sharp transitions were observed at 1.0-2.0 M with no detectable aggregates being formed beyond 2.0 M (Fig 4.9B). In GdHCl, the detectable aggregation was slowed down noticeably at 0.5 M with no aggregate formation at concentrations of 0.75 M or beyond (shown later in section 4.3.6).
Characterization of Xylanase in DMSO-Water Mixtures

**Fig. 4.9:** Light scattering profile of the aggregate formation at 60 °C monitored by UV absorbance at 360 nm. **A.** as a function of time in varying DMSO concentrations, **B.** as a function of DMSO concentration after the saturation phase (i.e., 15 min).

DMSO has been reported to induce aggregation in rabbit muscle creatine kinase and other proteins (Ou, *et al.*, 2002b; Tjernberg, *et al.*, 2006) and thus the protection offered by DMSO against heat induced denaturation of xylanase was in contrast to the earlier reported effect of DMSO on other proteins. DMSO appears to reverse this aggregation process. However it is not clear whether this effect is due to stabilization of the protein or simply due to dissolution of the aggregate. Hence we decided to monitor the effects of DMSO on thermal inactivation of xylanase by activity measurements.

Xylanases are reported to undergo irreversible denaturation with increase in temperature primarily due to the aggregation of the protein (Davoodi *et al.*, 1998). The time-dependent temperature induced deactivation of enzymes is a known phenomenon and many enzymes undergo such denaturation at higher temperatures. The transition against the thermal induced aggregation was observed in the region 10-15 % (v,v) DMSO (Fig. 4.9 B) and thus thermal inactivation measurements were made at DMSO concentration (v,v) of 0 % and 12 % (i.e., in aqueous condition and at [C]_{1/2} value of DMSO, section 4.3.1).
The half life of deactivation in presence of 12 % DMSO increased to ~ 4 min from ~ 2 min in aqueous solution (Fig. 4.10). Thus it appears that DMSO might be offering a certain level of stabilization of the protein in addition to the dissolution of aggregates.

**Fig. 4.10:** Thermal inactivation kinetics of xylanase in, A). 0 % DMSO and B). 12% DMSO-water mixture.

Xylanases has potential application in pulp and paper industry where it is used to dechlorinate the pulp and the process is carried out at higher temperatures. However, at higher temperatures xylanases undergo an irreversible denaturation (Davoodi, *et al.*, 1998) due to the aggregation of the molecule. Numerous attempts have been made to stabilize the xylanases against the thermal induced denaturation and the studies involve random mutagenesis, gene shuffling, site specific mutation, directed evolution, N-terminus extension of the protein,
Characterization of Xylanase in DMSO-Water Mixtures

introduction of disulphide bridges in the protein molecule, and addition of polyols or simple salts (Arase, et al., 1993; Breccia, et al., 1998; Cobos and Estrada, 2003; Davoodi, et al., 1998; Fenel, et al., 2004; Georis, et al., 2000; George, et al., 2001; Paes and O'Donohue, 2006; Palackal, et al., 2003; Park, et al., 2001; Shibuya, et al., 2000; Stephens, et al., 2007; Turunen, et al., 2001; Turunen, et al., 2002; Wakarchuk, et al., 1994a; Xiong, et al., 2004). However, none of the above studies say anything about the temperature stability of xylanase in presence of non-aqueous solvents. Even though the possibility exists that a combination of solvent engineering and protein engineering methods may ultimately prove more successful in producing the "ideal" xylanase for industrial applications.

The effect of DMSO on the stability of xylanase was monitored using thermal and chemical denaturation studies. The thermal unfolding of xylanase is irreversible and largely due to the aggregation of the protein occurring during the transition period. However, the transition is reversible in presence of 2.5 M urea ((Davoodi, et al., 1998). Hence the unfolding studies were carried out in presence of 2.5 M urea in DMSO concentrations ranging from 0 % to 30 % of DMSO (v,v). The thermal unfolding transitions in aqueous and DMSO-water mixtures were obtained by UV thermal melting experiments. The raw data obtained after the thermal melts is shown in Fig. 4.11 A. Only slight changes in T_m values occurred with shifting from ~ 54 °C in 0 % DMSO to ~ 49 °C in 30 % DMSO (Fig. 4.11 B, Table 4.4). Also there was a noticeable change in the magnitude of difference between the pre-transition and post-transition baselines. The magnitude of change in absorbance as compared to that in 0 % DMSO (set as 100 %) progressively declined and showed an approximately linear correlation with the increasing concentration of the DMSO (Fig. 4.11 C). However this effect was due to perturbation of the absorption spectrum of the Trp residues of xylanase in DMSO (as observed for NATA, data not shown) The changes in the mid point of thermal unfolding transition (T_m) as a function of DMSO concentration is listed in table 4.3.
Characterization of Xylanase in DMSO-Water Mixtures

![Graphs A, B, and C](image)

**Fig. 4.11: A) and B).** Thermal denaturation of purified recombinant xylanase in varying concentrations of DMSO monitored by UV absorbance at 293 nm. **C).** Relative change in absorbance at 293 nm of the native (i.e., 25°C) and thermally unfolded state (i.e., 70°C) with reference to the change in 0% DMSO as 100.

<table>
<thead>
<tr>
<th>DMSO (v/v)</th>
<th>T_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>53.7</td>
</tr>
<tr>
<td>5%</td>
<td>53.6</td>
</tr>
<tr>
<td>10%</td>
<td>53.3</td>
</tr>
<tr>
<td>15%</td>
<td>53.1</td>
</tr>
<tr>
<td>20%</td>
<td>51.6</td>
</tr>
<tr>
<td>30%</td>
<td>49.1</td>
</tr>
</tbody>
</table>

**Table 4.3:** Variation of transition mid point of thermal unfolding (T_m) with DMSO concentrations.
4.3.5 Circular Dichroism of Xylanase in DMSO-Water Mixtures

In order to further monitor the effects of DMSO on the conformation of xylanase near-UV CD spectra were recorded from 250-320 nm. Increasing DMSO concentration showed a progressive around 250-320 nm (Fig 4.12A).

Near UV CD spectra of proteins are, in general are not amenable to direct interpretations in terms of protein structure (Kahn, 1979). However they are sensitive to differences in tertiary structure of related proteins or of the same protein in different environments. Near UV CD spectra of protein is largely due to the conformation of aromatic amino acids in a given environment. Normally Tyr has peak closer to 290 nm with fine structure between 290-305 nm; Trp has peak between 275 and 285 nm and Phe having sharp fine structures between 255-270 nm (George and Phillips, 2007). The alterations in peak position or shape correspond to the perturbations of the microenvironment of the corresponding amino acids in the protein. The difference spectra of the xylanase in 0 % and 50
Characterization of Xylanase in DMSO-Water Mixtures

% DMSO at 25 °C (data not shown) showed positive peaks at 284, and 291 nm and negative peaks at 278, 287 and 296 nm indicating significant perturbations around the aromatic amino acids in xylanase. Xylanase possess 11 Trp, 16 Tyr and 4 Phe spread over the entire protein. This indicates that DMSO induced perturbation are global in nature and cannot be localized to the region around one or few amino acids.

The far-UV CD spectrum (200-260 nm) of the xylanase in 0 % DMSO didn’t show any structural perturbations in temperature range up to 40 °C. The spectrum at 25 °C and 40 °C was similar. Also the spectrum recorded after cooling of the sample to 25 °C from 40 °C was super imposable to that obtained earlier (Fig 4.12B). Although it is easy to monitor the changes in the secondary structure region using far-UV CD, the high absorbance of DMSO at lower wavelengths precluded the monitoring of any changes with increasing DMSO concentrations.

![Graph showing far-UV CD spectra of the purified recombinant xylanase.](image)

Fig 4.12: B). Far-UV CD spectra of the purified recombinant xylanase. The ellipticity scale is in deg.cm².dmool⁻¹.
4.3.6 Effect of DMSO on the Chemical Denaturation of Xylanase

As observed for DMSO (section 4.3.4) the commonly used denaturants GdHCl and urea also offered a protection to xylanase against heat induced denaturation. Fig. 4.13 shows the effect of these denaturants on the aggregation behavior of xylanase.

![Graph A](#)  
![Graph B](#)  
![Graph C](#)

**Fig. 4.13:** Light scattering profile of the aggregate formation at 60 °C monitored by UV absorbance at 360 nm. **A).** as a function of time in varying urea concentrations, **B).** as a function of time in varying GdHCl concentrations, **C).** as a function of DMSO concentration after the saturation phase (i.e., 15 min).

To further probe the conformational stability of xylanase, chemical denaturation studies were carried out using GdHCl or urea induced unfolding titrations. The unfolding titrations of xylanase in presence of 0 % DMSO at lower GdHCl concentrations showed an increase in fluorescence accompanied by red shift at higher concentrations. The complete unfolding was observed at GdHCl concentrations of 2.5 M or beyond (Fig. 4.14A). The increase in fluorescence
intensity at lower concentrations with red shift of the spectrum at higher concentrations was also observed in urea induced unfolding transition. The complete unfolding in urea was observed beyond 6.0 M (data not shown).

The relative fluorescence intensity of tryptophans shows dependency on urea concentration which is much more at higher concentrations. However in GdHCl no such dependency is seen up to a concentration of 5.0 M (Creighton, 1997). Hence DMSO effects were studied in GdHCl induced unfolding transitions. The increase in fluorescent intensity at low GdHCl concentrations was also seen in the presence of DMSO (Fig. 4.14 B-D), accompanied by red shift at higher concentrations as observed in 0 % DMSO (Fig. 4.14A). The $\lambda_{\text{max}}$ for the emission spectrum of xylanase in the completely unfolded state (i.e. in 5.0 M GdHCl) changed from ~357 in 0 % DMSO nm to ~355 nm in 20 % DMSO.

![Fluorescence spectra of purified recombinant xylanase in varying GdHCl concentrations in presence of A). 0 % DMSO, B). 5 % DMSO, C). 10% DMSO and D). 20 % DMSO. Samples were equilibrated overnight (16-18 hrs. prior to spectral measurements.](image)

**Fig. 4.14:** Fluorescence spectra of purified recombinant xylanase in varying GdHCl concentrations in presence of A). 0 % DMSO, B). 5 % DMSO, C). 10% DMSO and D). 20 % DMSO. Samples were equilibrated overnight (16-18 hrs. prior to spectral measurements.
Characterization of Xylanase in DMSO-Water Mixtures

The changes in average emission wavelength ($<\lambda>$) of xylanase as a function of GdHCl concentrations in varying DMSO concentration is shown in Fig. 4.15 A. The mid point of the unfolding transition changed from 1.62 M in 0% DMSO to 1.33 M in 20% of DMSO. The average emission wavelength ($<\lambda>$) in 0% DMSO changed from ~356 nm in the native state (pre-transition baseline) to ~366 nm in the unfolded state (post-transition baseline). The changes in 20% DMSO were from ~351 nm to ~364 nm (Fig. 4.15 A). The unfolding data was analyzed according to the two state model (as described in section 4.2.8) and the resulting denaturation parameters are listed in table 4.4. The variation of $\Delta G$ values for the unfolding of xylanase in varying concentration of DMSO is shown in Fig. 4.15 B. The slope of change of average emission wavelength of unfolded xylanase (at 3.0, 4.0 and 5.0 M GdHCl) as a function of DMSO concentration was parallel to NATA (Fig. 4.15 C ) indicating maximum solvent exposure of all the tryptophan residues and thus complete unfolding in DMSO-water mixtures.

<table>
<thead>
<tr>
<th>DMSO</th>
<th>$[D]_{1/2}$</th>
<th>m</th>
<th>$\Delta G_d(H_2O)$</th>
<th>$\Delta \Delta G_d(H_2O)$</th>
<th>$\Delta[D]_{1/2}$</th>
<th>$\Delta (\Delta G)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>1.62</td>
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</tr>
<tr>
<td>5%</td>
<td>1.61</td>
<td>4.097</td>
<td>6.62</td>
<td>0.85</td>
<td>0.01</td>
<td>0.016</td>
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<td>5.25</td>
<td>2.22</td>
<td>0.08</td>
<td>0.126</td>
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<tr>
<td>20%</td>
<td>1.33</td>
<td>2.961</td>
<td>3.99</td>
<td>3.48</td>
<td>0.29</td>
<td>0.428</td>
</tr>
</tbody>
</table>

Table 4.4: Comparative profiles for the free energy of denaturation of xylanase by GdHCl in varying concentrations of DMSO.

a $[D]_{1/2}$ is mid point of the GdHCl unfolding curve in molar M).
b The slope of the extrapolated curve in kcal mol$^{-1}$ M$^{-1}$. (see eqn ***)
$\Delta G_d(H_2O)$ Free energy of unfolding in absense on any denaturant.
d Difference between $\Delta G_d(H_2O)$ values in kcal mol$^{-1}$.
e Difference between the $\{GdHCl\}_{1/2}$ values in molar (M).
f The product of $\Delta[D]_{1/2}$ and average of the two m values.
Characterization of Xylanase in DMSO-Water Mixtures

Fig. 4.15: Spectral changes of xylanase in presence of varying concentrations of DMSO A). Change in average emission wavelength as a function of GdHCl concentration, B). \( \Delta G \) for xylanase unfolding as a function of GdHCl concentration, C). Change of average emission wavelength of unfolded xylanase and NATA as a function of DMSO.

Both \( m \) value and \( \Delta G_d(H_2O) \) for GdHCl denaturation decrease linearly with the concentration of DMSO (Fig. 4.16 C & D) indicating that both destabilizers (i.e., GdHCl and DMSO) act independently on the protein without producing any synergistic/antagonistic effects. Overall the results indicated a decrease in the stability with an increase in DMSO concentration. The changes in surface tension values have been correlated with stabilization/destabilization effects of the added co-solvent (Arakawa and Timasheff, 1982, Lee and Lee, 1981). Studies on the effect of changes in surface tension of the DMSO-water mixtures have been reported to behave in a non-ideal manner (Markarian and Terzyan, 2007). However in the concentration range of DMSO used in our studies, the surface tension values
shows a decrease. Decrease in surface tension thus accounts well for the observed loss in the stability of the xylanase in DMSO-water mixtures. The change of $[D]_{1/2}$ and $T_m$ (Fig. 4.16 A & B) also show a decline which is in agreement with the changes of surface tension values for DMSO-water mixtures in the concentration range (0-20 %) used in current study.

![Graphs A, B, C, D showing changes in stability parameters](image)

**Fig. 4.16:** Effect of DMSO on the stability parameters, A). transition mid point of thermal denaturation $T_m$, B). transition midpoint of chemical denaturation $[D]_{1/2}$, C). $m$ value and D). Free energy of unfolding $\Delta G_d(H_2O)$.

### 4.3.7 ANS Binding to Xylanase

Protein bound ANS fluorescence has been traditionally used as probe for changes in protein conformation upon partial denaturation (Stryer, 1965). However xylanase didn’t show binding of ANS up to 100 $\mu$M of ANS at pH 7.0 even in the presence of 30 % DMSO and to a completely unfolded state in 6.0 M
Characterization of Xylanase in DMSO-Water Mixtures

guanidine hydrochloride. The results are in agreement with the previous studies (Nath and Rao, 2001b; 2001c, Nath, et al., 2002). This behavior of xylanase is quite unusual and could be due to the relative paucity of charged amino acids on the protein surface that is required for ANS binding in addition to the hydrophobic patches (Matulis and Lovrien, 1998). Earlier reports also have shown that ANS binding to xylanase is highly selective and occurs in a very narrow range of ~ pH 1.8 where it forms a molten globule like state (Nath and Rao, 2001c). Since in this pH region, xylanase doesn’t exist in its native form, it was not of critical importance to study the effects of DMSO on xylanase at this pH. Hence further binding studies of ANS with xylanase in presence of DMSO was not carried out.

4.3.8 Time Resolved Fluorescence Measurements

Time resolved fluorescence was used as an independent monitor of the conformational environment of Trp residues in xylanase. The 11 tryptophan residues of xylanase are located in different environments and differ in their relative accessibility. The excited state lifetimes are distinct for the tryptophanyl residues located in dissimilar environments and also the perturbation to the microenvironments of these tryptophans by the DMSO is certainly not uniform. Deconvoluting of the fluorescence decay curves in terms of the possible multi-exponential process for each of the 11 tryptophans would be extremely difficult. Hence we decided to interpret the fluorescence decay curves in terms of continuous rather discrete components. In this model all the tryptophans of the protein are assumed to exist in one or few conformational environments, where each such environment is characterized by a continuous distribution of fluorescence lifetimes with distinct averages and standard deviations. Fig. 4.17
Fig 4.17: Life time distribution analysis of time resolved fluorescence intensity decay curves of xylanase in various conditions. A). 0 % DMSO, B). 5 % DMSO, C). 10 % DMSO, D). 20 % DMSO, E). 30 % DMSO and F). 6.0 M GdHCl
Characterization of Xylanase in DMSO-Water Mixtures

shows the life time distributions of the Trp residues of xylanase in absence and presence of DMSO. In absence of DMSO at 25 °C three distinct classes of fluorophores (Fig. 4.17 A) appear in the life time distribution: one short lived (mean lifetime $\langle \tau_1 \rangle$) centered around 1.08 ns, middle component ($\langle \tau_2 \rangle$) centered around 3.45 ns and the long lived component ($\langle \tau_3 \rangle$) centered around 7.18 (Fig. 4.17, Table 4.5). The middle component $\langle \tau_2 \rangle$ in xylanase (in absence of DMSO, i.e., 0 % DMSO) probably reflects the surface exposed tryptophan residues as it is closest to that of NATA (data not shown).

One can estimate the number of tryptophans in each environment from the relative area within each peak. The calculated number of tryptophan in each environment as a function of DMSO concentration is given in Table 4.5.

| Xylanase | $\langle \tau_1 \rangle$ | $f_1$ | Trp$_1$ | $\langle \tau_2 \rangle$ | $f_2$ | Trp$_2$ | $\langle \tau_3 \rangle$ | $f_3$ | Trp$_3$ | $\chi^2$
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
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<tbody>
<tr>
<td>0 % DMSO</td>
<td>1.086</td>
<td>32.81</td>
<td>3.6</td>
<td>3.455</td>
<td>22.40</td>
<td>2.46</td>
<td>7.181</td>
<td>44.56</td>
<td>4.9</td>
<td>1.095</td>
</tr>
<tr>
<td>5 % DMSO</td>
<td>0.944</td>
<td>31.49</td>
<td>3.46</td>
<td>2.773</td>
<td>22.36</td>
<td>2.46</td>
<td>7.077</td>
<td>46.15</td>
<td>5.07</td>
<td>1.242</td>
</tr>
<tr>
<td>10 % DMSO</td>
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<td>-</td>
<td>-</td>
<td>1.195</td>
<td>42.99</td>
<td>4.73</td>
<td>6.103</td>
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<td>-</td>
<td>-</td>
<td>1.269</td>
<td>45.03</td>
<td>4.95</td>
<td>6.115</td>
<td>54.97</td>
<td>6.04</td>
<td>1.316</td>
</tr>
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<td>30 % DMSO</td>
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<td>5.769</td>
<td>55.11</td>
<td>6.0</td>
<td>1.429</td>
</tr>
<tr>
<td>6.0 M GdHCL</td>
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<td>2.612</td>
<td>98.79</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.113</td>
</tr>
</tbody>
</table>

Table 4.5: Life time distribution of xylanase in absence and presence of DMSO and 6.0 M GdHCl at 25 °C. $\langle \tau \rangle$ is the mean lifetime of fluorescent intensity decay, $f$ is the relative fractional intensity of each class of fluorophores, and $\chi^2$ defines the goodness of the fitting values. Trp$_1$, Trp$_2$ and Trp$_3$ are the number tryptophan residues in each distinct environment as a function of DMSO concentration.
Examination of the table shows that in the absence of DMSO, the middle component $\tau_2$ (which appears to due to surface exposed tryptophans) comprises of ~3 tryptophan residues. We calculated the relative surface exposure of all the 11 tryptophans in the crystal structure of native xylanase (Table 4.6). The data shows that Trp 9, Trp 185 and Trp 6 are the three most solvent exposed tryptophans. These are the most likely residues that contribute to the lifetime component 2. Examination of the xylanase structure (Fig. 4.18) shows that Trp 9 is close to the tip of the fingers domain, Trp 185 is the C-terminal residue and Trp 6 exists near to the boundary of sheet I in the finger domain.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Exposed surface Area (Å²)</th>
<th>Relative accessibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp 6</td>
<td>69.475</td>
<td>27.36</td>
</tr>
<tr>
<td>Trp 9</td>
<td>90.685</td>
<td>36.61</td>
</tr>
<tr>
<td>Trp 30</td>
<td>6.959</td>
<td>2.69</td>
</tr>
<tr>
<td>Trp 42</td>
<td>13.003</td>
<td>5.09</td>
</tr>
<tr>
<td>Trp 58</td>
<td>34.819</td>
<td>13.37</td>
</tr>
<tr>
<td>Trp 71</td>
<td>6.175</td>
<td>2.36</td>
</tr>
<tr>
<td>Trp 85</td>
<td>20.241</td>
<td>8.28</td>
</tr>
<tr>
<td>Trp 129</td>
<td>15.051</td>
<td>5.79</td>
</tr>
<tr>
<td>Trp 153</td>
<td>0.580</td>
<td>0.21</td>
</tr>
<tr>
<td>Trp 164</td>
<td>45.130</td>
<td>17.44</td>
</tr>
<tr>
<td>Trp 185</td>
<td>87.833</td>
<td>28.06</td>
</tr>
</tbody>
</table>

Table 4.6: Exposed surface area and relative accessible surface area of tryptophan residues in crystal structure of xylanase 1XNB. Relative accessible surface area is defined as ratio of surface accessibility of residue ‘X’ to surface accessibility of residue ‘X’ in a model peptide Gly-X-Gly in a backbone conformation identical to that of the residue ‘X’ in the native crystal structure.

With increasing concentration of DMSO all the three peaks broaden appreciably (Fig.4.17 A-E) indicating that the microenvironment around each of the Trp residues become more heterogeneous. At higher concentrations the peak broaden appreciably and begins to merge with each other, with peaks 1 and 2
merging at DMSO concentration 5-10 % (v,v). In a control experiment where xylanase was denatured with 6.0 M GdHCl, the life time measurements shows a very broad single peak (Fig.4.17F), indicating that all the tryptophans are in similar but highly heterogeneous environment.

Fig. 4.18: Structural model of xylanase showing the relative location of different tryptophanyl residues in crystal structure of xylanase 1XNB. The most accessible tryptophans (Trp 6, 9, 185) are marked. The progressive change in color of the tryptophans from white to red indicates the gradual decrease in their relative accessibility surface area.

The gradual broadening and merging of the peaks with increasing DMSO concentration (Fig.4.17 A-E) indicate that the corresponding microenvironment around the tryptophan residues gradually lose their distinct identity. The process
is not complete even in 30 % DMSO, but found to be complete when xylanase was denatured with 6.0 M GdHCl. The results are consistent with earlier studies on protein-DMSO interaction (Jackson and Mantsch, 1991) which shows that most proteins are completely denatured at high concentrations of DMSO.

However the interaction of xylanase with low concentrations of DMSO is interesting for a number of reasons. Firstly, kinetic measurements show an increase in the $V_{\text{max}}$ in presence of low DMSO. There is also a reduction in the pH optimum of the xylanase. This shows that at low concentration DMSO seems to influence the mechanism of xylanase in some way other than simple structural perturbations. Secondly, xylanase seems to be kinetically stabilized to an extent against temperature induced denaturation. This could be due to the alteration in the temperature dependent denaturation mechanism of the enzyme. The overall observed effects provided global information of the behavior of xylanase in DMSO-water mixture but did not provide any detailed information on mechanism of action of DMSO on the structure or dynamics of the protein. Thus it was further decided to carry out the molecular dynamics simulation of xylanase in presence and absence of DMSO to gain mechanistic insight into the behavior of xylanases in DMSO-water mixtures.