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# Mechanism of stabilization of *Bacillus circulans* xylanase upon the introduction of disulfide bonds

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**Abstract**

The introduction of disulfide bonds has been used as a strategy to enhance the stability of *Bacillus circulans* xylanase. The transition temperature of the S100C/N148C (DS1), V98C/A152C (DS2), and A1GC/G187,C188 (cXl) in comparison to the wild type was increased by 5.0, 4.1 and 3.8 °C, respectively. Interestingly, a combination of two disulfide bonds of DS1 and cXl (cDS1, circular disulfide 1) led to a 12 °C increase in the transition temperature. Importantly, an increase in the melting point and $\Delta\Delta G$ values of the cDS1 mutant was cooperative. These results suggest that the mechanism of stabilization by disulfide bonds under irreversible denaturation condition is achieved through: (1) a change in the rate-limiting step on the denaturation pathway; (2) destabilizing the unfolded state without affecting the relative rate constants on the denaturation pathway (like cXl mutant); and (3) or combination of the two (cDS1 mutant).


*Keywords:* Xylanase; Stabilization; Disulfide bond

## 1. Introduction

Xylanases (xylanohydrolases) are a class of enzymes involved in the hydrolysis of the xylan, the second most abundant polysaccharide in nature. The degradation of β-1,4 xylans involves xylanolytic systems, which include β-1,4 xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). The former is generally considered to hydrolyze the xylan backbone whereas the latter enzyme is implicated in the hydrolysis of the xylo-oligomers [1]. This ability renders xylanases a valuable tool in various biotechnology applications ranging from the food industry to pulp and paper industry [2]. The most important application of xylanases is in the paper industry, which is used for the selective hydrolysis of hemicelluloses in the pulp bleaching process. This application, however, depends upon thermal stability of the proteins. It has been shown that introducing disulfide bonds can substantially enhance thermal stability of proteins [3–9]. The stabilizing effect of a covalent cross-link is usually assumed to arise from its destabilizing effect on the unfolded state, by decreasing its conformational entropy [10,11]. This is because the presence of a covalent cross-link limits the motional freedom of the protein backbone in the unfolded state, thereby decreasing the entropy. In contrast to other interactions, for instance hydrogen bonds and electrostatic interactions, formation of a disulfide bridge as a covalent bond requires strict stereochemical conditions regarding the relative positions and orientations of the two participating cysteine residues [10]. The difficulties in designing new disulfide bonds inside proteins can be overcome by comparing homologous proteins. A comparison of the low molecular weight xylanase A sequence from *Schizophyllum commune* with the xylanase from *Bacillus circulans* revealed the presence of a disulfide bridge between positions 111 and 160 in the former, which corresponds to positions 100 and 148 in the latter [12]. Therefore, a disulfide bond was introduced at this position yielding DS1 mutant (Fig. 1). With the additional idea that a cross-link in the center of

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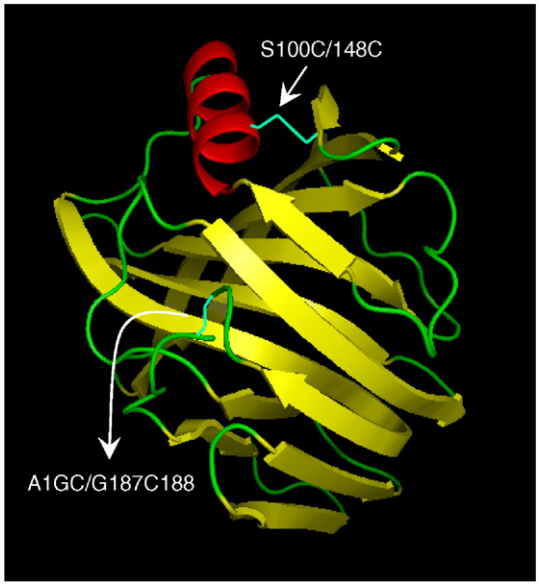


Fig. 1. Cartoon view of *B. circulans* xylanase with disulfide bonds shown in cyan.

the α-helix (instead of a S–S bond located at the edge of the α-helix as in the DS1 mutant) may be more effective in enhancing xylanase stability, another disulfide bridge was established between residues 98 and 152 (DS2). Furthermore, based on the suggestion that the increase in stability of a protein due to the introduction of a disulfide bond is proportional to the number of residues linked by the S–S bond [13], we decided to link C-terminus to the N-terminus through a disulfide. This was possible because in *Bacillus circulans* xylanase, the N-terminus is located in proximity to the C-terminus. The effect of these mutations on thermal stability, i.e. thermal transition temperature, conformational stability, i.e. $\Delta G_d(H_2O)$ as well as the mechanism of stabilization of *B. circulans* xylanase under reversible as well as irreversible conditions was investigated.

## 2. Materials and methods

Unless otherwise stated, materials used in this study were purchased from the Sigma Chemical Company. The bacterial strains as well as procedures used for site-directed mutagenesis and protein expression have been described in detail elsewhere [14–16].

### 2.1. Protein purification

In an ice bath, a mixture of cell paste and alumina was ground at least for 15 min. While being mixed, extraction buffer (10 mM sodium acetate, pH 5.3) and DNase (with a final concentration of 20 μg/mL) were added. The mixture was centrifuged at 6000 rpm for 20 min. The supernatant was further centrifuged at 10 000 rpm for 30 min. The purification was continued by exhaustive dialysis of the supernatant against 6 l of extraction buffer for 24 h using a mco 3500 Spectropor 3 dialysis bag (Canlab, Mississauga, Ontario). After centrifugation for 30 min at 10 000 rpm, the supernatant was separated and loaded onto a Poros HS II column (Perseptive Biosystems, Cambridge, MA) pre-equilibrated with 10 mM sodium acetate, pH 5.3. The eluted enzymes were further purified using Toyopearl resin column (HW 50-s, 1000–90 000 MW, Supelco Canada, Mississauga, Ontario). The concentration of the eluted xylanase or its mutants was determined from the absorbance of 280 nm, using an extinction coefficient of $A_{280}^{1\ mg/mL}=4.07$.

### 2.2. Enzyme activity

Enzyme activity was measured based on the reaction of reducing sugars with hydroxybenzoic acid hydrazide reagent [17]. Experimental mixtures contained 7 mg/mL soluble fraction of Birchwood xylan and 4 μg/mL of enzyme in assay buffer (50 mM sodium citrate, pH 5.5). Following 3 min of incubation at 40 °C, 50 μL of the mixture was mixed with 1 mL of 10 mM NaOH to stop the hydrolysis. Hydroxybenzoic acid hydrazide reagent with a final concentration of 0.05 M was added to the solution. To develop the colour, samples were immersed in boiling water for 10 min. The intensity of the color resulting from the reaction was measured at 420 nm.

### 2.3. Differential scanning calorimetry

Calorimetric thermograms were obtained using a MC-2 Microcal differential scanning calorimeter. Protein solutions were prepared in ammonium acetate buffer, pH 6.0, at a concentration of 0.5–1 mg/mL. All solutions were degassed under vacuum. The reversibility of the thermally induced transitions was always checked following the first run by re-heating the solution in the calorimeter cell immediately after cooling. Baselines obtained by filling both cells with the corresponding buffer for each particular condition were subtracted from the sample experimental trace, thus giving the apparent heat capacity profile. The transitions were corrected for the minor difference in heat capacity between the initial and the final states, using the Progress baseline procedure. The resulting curve, after normalization to the concentration of protein, is called the excess heat capacity profile. Data treatment was performed using the Origin software provided by the manufacturer of the calorimeter (Microcal, Inc.). The enthalpy of denaturation was determined by integration of the heat capacity function. Instrumental response correction based on the procedure developed by Lopez-Mayorga and Freire [18] had a negligible effect on the shape of calorimetric thermograms (data not shown).

### 2.4. Circular dichroism spectroscopy

The CD spectra of xylanase proteins were obtained using a JASCO J-600 spectropolarimeter as described previously [19]. Spectra in the near UV region were measured in a 1-cm path length cylindrical quartz cell with a protein concentration of about 0.3–0.5 mg/mL in either 50 mM ammonium acetate or 20 mM phosphate buffer. Spectra in the far-UV region were measured in a 0.02-cm path length cylindrical quartz cell with a similar protein concentration in 20 mM phosphate buffer. The

normalization of spectra was performed using the software provided by the instrument manufacturer. The molecular weight of the wild type protein was considered to be 20,397 Da. Except for the case of cXl and cDS1 mutants, small variations in the molecular weight due to mutations were ignored during the normalization of the mutants' spectra.

### 2.5. Chemical denaturation experiments

Guanidine hydrochloride or urea promoted unfolding was monitored by circular dichroism spectroscopy at 228 nm. For each point on the denaturation profile, 20 μL solution of the enzyme was diluted in a 180 μL mixture of the appropriate amount of denaturant and buffer and incubated for about 15 h at 24 °C. The free energy of unfolding by guanidine hydrochloride is calculated from the following equation [20]:

$$\theta_{228} = \frac{\theta_F + \theta_U \exp\left\{\frac{m[D]-\Delta G_d(H_2O)}{RT}\right\}}{1+\exp\left\{\frac{m[D]-\Delta G_d(H_2O)}{RT}\right\}} \quad (1)$$

where [D] is the concentration of denaturant, $m$ is a measure of dependence of $\Delta G$ on denaturant concentration, $\theta_F$ and $\theta_U$ are the values of the CD signal of the folded and unfolded forms of the protein. Using Origin software, experimental data were directly fitted to Eq. (1) in order to obtain the values of $m$ and $\Delta G_d(H_2O)$. The values of $\theta_F$ and $\theta_U$ at any concentration of denaturant can be obtained by linear extrapolation of the two plateau regions.

## 3. Results

### 3.1. Characterization of the mutants

Prior to any assessment of the stability achieved by site directed mutagenesis, it is essential to determine whether or not different variants of the xylanase are properly folded. Therefore, the secondary and tertiary structures of wild type xylanase and its variants were compared using far- and near-UV circular dichroism spectroscopy. The secondary structure of all of the mutants remained essentially unchanged, since no major differences was seen in the far-UV circular dichroism spectra of the mutants compared to wild type (Fig. 2). Furthermore, except for the DS2 mutant, the near-UV CD spectra of the wild type and mutants appeared to be identical. The near-UV CD spectrum results from the near-UV electronic transitions of aromatic side chains, and their coupling with electronic transitions arising from aromatic residues, peptide bonds, and charged groups in an asymmetric environment [21]. Therefore, any change in the environment of these residues, due to change in tertiary structure, would affect these electronic transitions leading to a different spectrum. Accordingly, identical near-UV CD spectra indicate that the tertiary structure of the mutants remained intact. Only in the case of the DS2 variant, elipticity was increased with respect to the wild type indicating a somewhat altered tertiary structure (Fig. 2). To insure that the difference in the spectrum is not due to an error in determination of protein

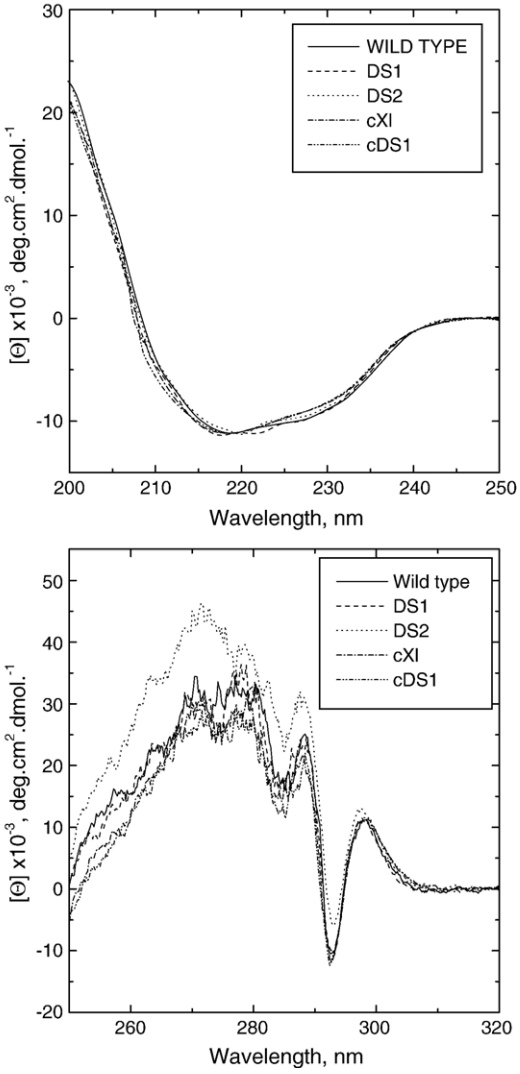


Fig. 2. Far-, upper panel, and near-UV, lower panel, circular dichroism spectra of the wild type xylanase and its disulphide bond mutants in 50 mM ammonium acetate buffer at pH 6.0.

concentration, the intensity of the spectrum resulting from DS2 mutant was multiplied by a fixed factor to determine if it would overlap with the spectrum from the wild type protein. Although this resulted in a spectrum that roughly overlapped with that of the wild type between 285 and 250 nm, a higher degree of deviation occurred at 293 nm indicating that the difference in the spectra of the wild type and DS2 variant is not due to any error in determination of protein concentration, rather it is due to structural alteration of the mutant protein. Overall, the circular dichroism data indicate that these mutations do not hamper proper folding of the proteins.

All of the mutants maintained high enzymatic activity (Table 1) with the exception of cDS1 variant. This mutant contains two disulfide bonds with identical positions to those in DS1 and cXl. When the disulfide bonds were introduced singly, as the DS1 or cXl variants, enzymatic activity remained intact. However, simultaneous introduction of the two was accompanied by 32% decrease in activity. Considering the fact that the simultaneous introduction of two disulfide bonds did

Table 1
The position of disulfide bonds introduced into *B. circulans* xylanase and their relative activities and melting points obtained by differential scanning calorimetry with a scanning rate of approximately 57 °C/h in 50 mM ammonium acetate buffer

| Protein | Amino acid changes | Description | Relative activity | $t_m$ (°C) | $\Delta t_m$ [a] |
|---|---|---|---|---|---|
| Wild type | None | No disulfide bond is present in the structure | 100 | 59.7 | N.A. |
| DS1 | S100C/N148C | Single disulfide bond mutant | 110 | 64.7 | 5.0 |
| DS2 | V98C/A152C | Single disulfide bond mutant | 95 | 63.8 | 4.1 |
| cXl | A1GC [b]/G187 [c], C188 | C-terminal is joined to N-terminal | 96 | 63.5 | 3.8 |
| cDS1 | S100C/ N148C,A1GC/ G187, C188 | Combination of DS1 and cXl mutations | 68 | 72.0 | 12.3 |

[a] $\Delta t_m$ is equal to the difference between the transition temperature of the mutant and wild type.
[b] The N-terminus was changed by the deletion of alanine1 and the addition of glycine and cysteine.
[c] The residues 187 and 188 were additions to the C-terminus with the numbering based on the N-terminal glycine being residue 1.

Table 2
A comparison of the melting points of the various mutants of *B. circulans* xylanase under reversible conditions (in 2.5 M urea) with those under irreversible conditions (in ammonium acetate buffer) obtained by differential scanning calorimetry, at two scanning rates of approximately 10 and 60 °C/h

| | Buffer | | | Urea | | |
|---|---|---|---|---|---|---|
| | $t_m$ ($v$=10) | $t_m$ ($v$=60) | $\Delta t_m$ [a] | $t_m$ ($v$=10) | $t_m$ ($v$=60) | $\Delta t_m$ |
| Wild type | 57.4 | 59.6 | 2.2 | 51.1 | 54.0 | 2.9 |
| DS1 | 61.6 | 64.5 | 2.9 | 58.6 | 59.4 | 0.8 |
| DS2 | 61.1 | 63.8 | 2.7 | 54.4 | 55.9 | 1.5 |
| cXl | 60.7 | 63.5 | 2.8 | 55.3 | 58.1 | 2.8 |
| cDS1 | 69.2 | 72.0 | 2.8 | 63.7 | 65.5 | 1.8 |

[a] $\Delta t_m$ is equal to difference between the melting points, $t_m$, at two scanning rates of 10 and 60 °C/h.

not appear to alter the structure of the protein (Fig. 2), activity decrease may be a consequence of an increase in rigidity of structure or some minor local changes that cannot be detected by CD. Moreover, these observations confirm the results from circular dichroism studies that the mutant proteins are properly folded.

### 3.2. DSC studies of the wild type xylanase and its variants under irreversible denaturation conditions

To compare the effects of different substitutions on thermal stability, differential scanning calorimetry was applied. This technique can be used to determine the thermodynamic parameters of the thermal unfolding, when the process is reversible. The thermal denaturation of the wild type xylanase and its variants in

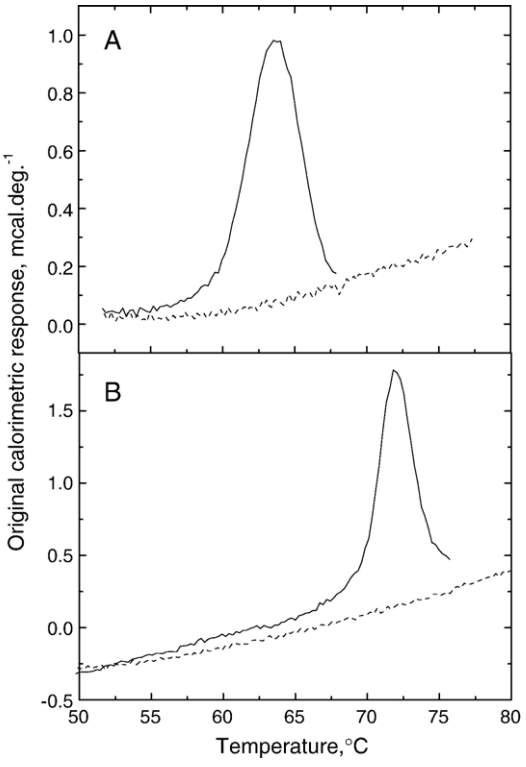


Fig. 3. Original calorimetric recordings for the cXl (A) and cDS1 (B) mutants of *B. circulans* xylanase at the scanning rate of about 58 °C/h in 50 mM ammonium acetate, pH 6.0, with protein concentrations of approximately 0.54 and 0.59 mg/mL, respectively. The dashed lines are the second heating run after cooling down the samples.

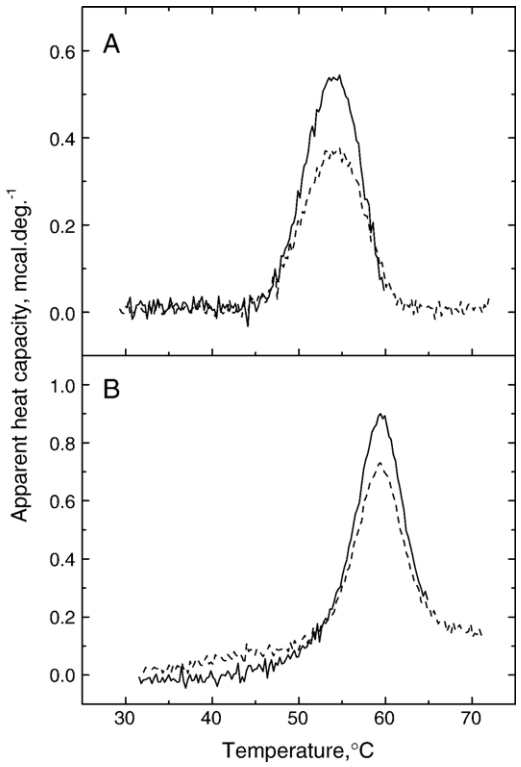


Fig. 4. Original calorimetric recordings for the wild type xylanase (A) and DS1 mutant (B) at the scanning rate of 58.0 °C/h in 2.5 M urea at pH 6.0 with protein concentration at about 0.5 and 0.6 mg/mL, respectively. The dashed lines are the second heating run after cooling down the sample.

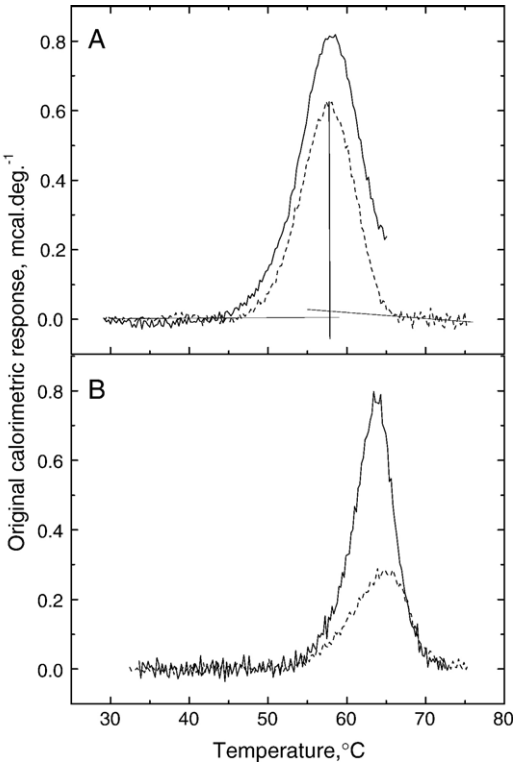


Fig. 5. Original calorimetric recording for the cXl mutant (A) and cDS1 mutant (B) with the protein concentrations of about 0.4 and 0.5 mg/mL in 2.5 M urea. The solid line shows the first heating run with the scanning rate of 58.0 °C/h and the dotted line is the second run with the scanning rate of 58 °C/h for the cXl mutant and 13 °C/h for cDS1 mutant after immediate cooling the sample of the proteins. Extrapolation of linear portions to $t_m$ on both sides of the DSC trace for cXl variant was used to obtain a rough estimate of the $\Delta C_p$ value.

buffer was found to be irreversible, as no transition was observed in the second run ([19] and Fig. 3). Consequently, thermodynamic parameters could not be determined under such conditions. Moreover, the transition temperature ($t_m$) of the wild type protein and its variants showed a dependence upon the rate of temperature increase (scanning rate). These facts indicate that the thermal denaturation is controlled by kinetic rather than thermodynamic factors. Nevertheless, for the sake of comparison of thermal stability achieved by mutations, the melting point ($t_m$) of the mutants was compared to wild type xylanase under conditions of identical scan rates. All of the mutants achieved considerable enhanced stability with respect to the wild type (Table 2). The cDS1 variant with a 12° increase in the melting point showed the maximum increased stability.

### *3.3. DSC studies of the wild type xylanase and its variants under reversible denaturation conditions*

As mentioned earlier, thermal denaturation of the wild type xylanase and its variants appeared to be irreversible due to aggregation. It has been shown that the aggregation of proteins can be prevented by denaturing agents like urea, or guanidine hydrochloride [22]. Hence, thermal denaturation of wild type and mutant xylanases was investigated in the presence of these agents. The presence of guanidine hydrochloride did not lead to a reversible thermal unfolding, as no transition was observed in the second heating run (data not shown). The presence of urea, however, prevented aggregation of all of the mutants leading to their reversible denaturation (Figs. 4 and 5). The extent of reversibility was found to be dependent upon concentration of urea. Since the reversibility of the thermal denaturation was improved up to 2.5 M urea, this concentration of urea was chosen to study reversible thermal denaturation of the wild type protein and its variants. To ensure that the presence of urea did not result in a major change in xylanase structure, circular dichroism spectra of the protein was measured at various urea concentrations. Fig. 6 shows that the CD spectra of the protein remain essentially unchanged between 0 and 5.5 M urea concentration. As the urea concentration was increased further, an abrupt change of the protein structure occurred. Furthermore, 2.5 M urea had a negligible effect on the enzymatic activity of the enzymes (data not shown). Therefore, conducting thermal denaturation in 2.5 M urea is justifiable, since under such conditions the proteins are far from the point where chemical denaturation takes place.

Under reversible conditions, thermodynamic parameters for the wild type xylanase and mutants were obtained by differential scanning calorimetry (Fig. 7). The area under each thermogram is equal to the calorimetric enthalpy ($\Delta H$) of thermal denaturation. Using the following equation, from the shift in $T_m$, the

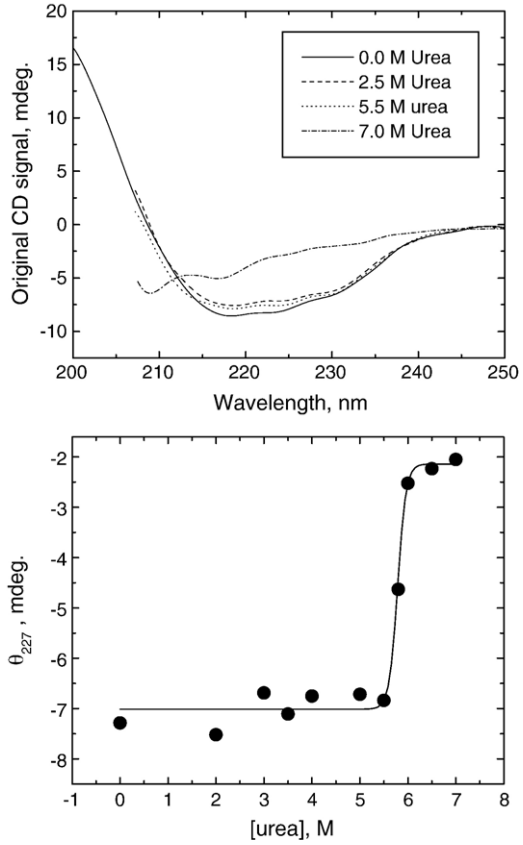


Fig. 6. Far-UV circular dichroism spectra of the wild type xylanase with a protein concentration of about 0.4 mg/mL in various concentrations of urea (A) and urea denaturation curve of the wild type xylanase monitored at 227 nm by circular dichroism spectroscopy (B).

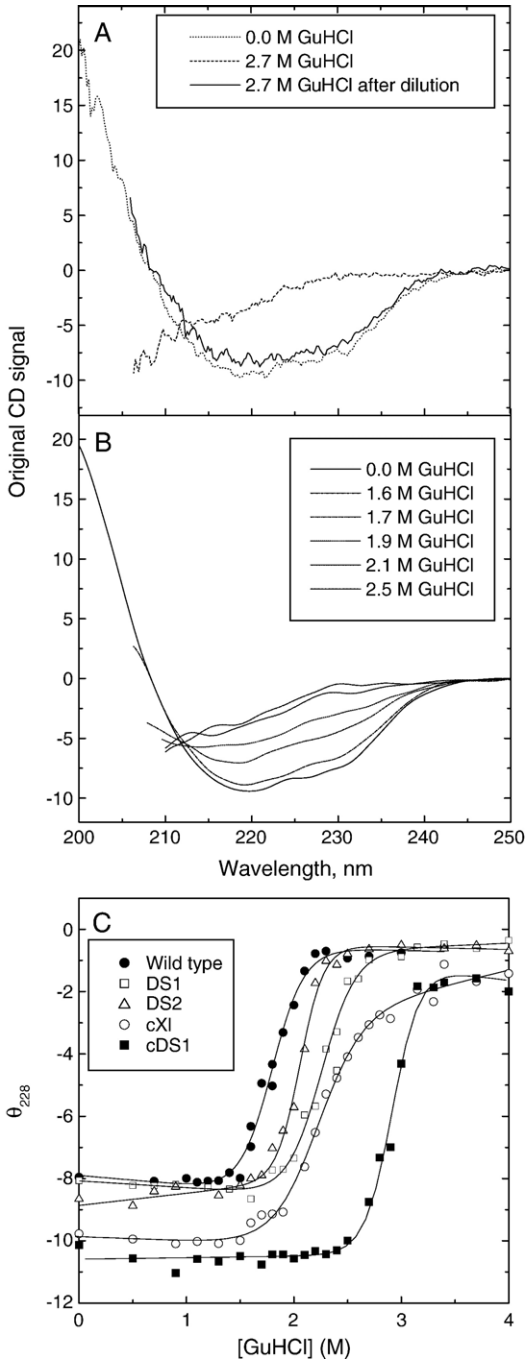


Fig. 7. Far-UV circular dichroism spectra of the wild type xylanase in various concentrations of guanidine hydrochloride (GdnHCl) with the protein concentration at about 0.5 mg/mL (A) reversibility of guanidine hydrochloride denaturation of the wild type xylanase verified by far-UV circular dichroism spectroscopy (B) and changes of CD signal at increasing concentrations of guanidine hydrochloride (C). A: The solid line is spectrum of the protein in the absence of GdnHCl with protein concentration of about 0.5 mg/mL, and the dashed line in the presence of 2.7 M GdnHCl measured in a 0.02-cm-path length cylindrical quartz cell. The dotted line is the latter sample after 50 times dilution with 20 mM phosphate buffer measured in a 1-cm path length cylindrical quartz cell. C: Guanidine hydrochloride denaturation curve of the wild type xylanase and its disulfide bond variants monitored at 228 nm wavelength by circular dichroism spectroscopy. The lines show the best fit of experimental data to Eq. (1).

changes in the apparent stability of mutants relative to the wild type, $\Delta(\Delta G)$ can be evaluated at the $T_m$ of the wild type protein:

$$\Delta(\Delta G) = \Delta G(\text{Mutant}) - \Delta G(\text{Wild Type}) \quad (2)$$

$\Delta G$(Wild Type) is zero at $T_m$, while the $\Delta G$(Mutant) can be obtained by Gibbs–Helmholtz equation. In order to prevent aggregation, the concentration of proteins was kept below 1 mg/mL. Consequently, the baseline stability of the instrument was not sufficient enough to allow for a reliable measurement of the heat capacity difference between the native and denatured states of the proteins. Nonetheless, we decided to obtain a rough estimate of the $\Delta C_p$ of the proteins by extrapolating the linear portions on both sides of the DSC traces to $t_m$ (as an example, see Fig. 5). The van't Hoff enthalpies of all of the mutants were found to be essentially identical to their calorimetric enthalpy. Therefore, thermal denaturation of the proteins obeyed a two-state reversible model [23]. The values of $\Delta(\Delta G)$ are listed in Table 3. A comparison of the melting temperature and $\Delta(\Delta G)$ of the wild type with those of the mutants shows a moderate stabilization of the DS2 and cXl mutants. On the other hand the increase of the melting point due to introduction of the disulfide bond in the DS1 protein is considerably more than in cXl and DS2. This is further supported by the comparison of the free energy of stabilization of mutants with respect to wild type (Table 3). The DS1 mutant with 3.0 kcal/mol free energy of stabilization is more stable than DS2 or cXl with 1.3 and 1.6 kcal/mol stabilization energies, respectively (Table 3). The sum of the $\Delta(\Delta G)$ of stabilization for cXl and DS1 is equal to 4.6 kcal/mol which is 0.8 kcal/mol less than that of the cDS1 mutant.

### 3.4. Guanidine hydrochloride denaturation of the wild type xylanase and its mutants

In order to gain further insight into conformational stability achieved by various mutations, chemical denaturation experiments were carried out using guanidine hydrochloride (GdnHCl) as a denaturant. To follow the effect of increasing concentration of guanidine hydrochloride on the structure of the proteins, far-UV circular dichroism spectroscopy was applied (Fig. 7). Maximal change in the spectrum of the wild type and mutant xylanase occurred in the wavelength range of 222–230 nm

Table 3
Thermodynamic parameters of the wild type xylanase and its disulfide bond mutants obtained from differential scanning calorimetry under reversible conditions (in the presence of 2.5 M urea)

| Protein | $\Delta H_{cal}$, kcal/mol | $t_m$, °C | $\Delta t_m$, °C | $\Delta C_p$, cal/mol/deg | $\Delta(\Delta G°)$, experimental kcal/mol | $\Delta(\Delta G°)$[a], theoretical kcal/mol |
|---|---|---|---|---|---|---|
| Wild type | 133 | 51.2 | 0 | 334 | 0 | 0 |
| DS1 | 138 | 58.6 | 7.4 | 410 | 3.0 | 4.5 |
| DS2 | 132 | 54.4 | 3.2 | 250 | 1.3 | 4.6 |
| cXl | 136 | 55.1 | 3.9 | 560 | 1.6 | 5.8 |
| cDS1 | 146 | 63.9 | 12.7 | 479 | 5.4 | 10.5 |

[a] These values were obtained from $\Delta S = -2.1 - (3/2)R \ln n$, in which $R$ is the gas constant and $n$ is the number of residues in the loop forming the disulfide bond.

Table 4
A comparison of free energy of denaturation of the disulfide bond mutants with that of wild type *B. circulans* xylanase obtained from fitting experimental data to Eq. (1) in GdnHCl denaturation experiments

| Protein | $[GdnHCl]_{50\%}$[a], M | *m*, kcal/mol/M | $\Delta G_d(H_2O)$ | $\Delta\Delta G_d(H_2O)$ | $\Delta G_d$[b](GdnHCl) | $\Delta\Delta G_d$(GdnHCl) |
|---|---|---|---|---|---|---|
| Wild type | 1.80 | 3.99±0.24 | 7.16±0.62 | 0.00 | −1.6 | 0.0 |
| DS1 | 2.25 | 3.71±0.17 | 8.37±0.71 | 1.21 | 0.2 | 1.8 |
| cXl | 2.20 | 2.84±0.11 | 6.25±0.60 | −0.91 | 0.0 | 1.6 |
| cDS1 | 2.92 | 4.68±0.20 | 13.7±1.0 | 6.54 | 3.4 | 5.0 |
| DS2 | 2.07 | 5.36±0.37 | 11.1±0.9 | 3.94 | −0.7 | 0.9 |

[a] $[GdnHCl]_{50\%}$ is the concentration of guanidine hydrochloride where 50% of protein is denatured.
[b] Free energy of denaturation in the presence of 2.2 M guanidine hydrochloride.

(data is shown for wild type only). Hence, any wavelength in this range can be used to follow the loss of the secondary structure as a function of guanidine hydrochloride concentration. The reversibility of denaturation was verified by diluting a concentrated GdnHCl solution of protein followed by measuring the CD spectrum (Fig. 7). Assuming unfolding follows a two state transition model, the equilibrium constant, *K*, between folded (F) and unfolded (U) states, $K=[U]/[F]$, and the free energy of unfolding, $\Delta G_U=-RT\ln K$, at a given concentration of GdnHCl were calculated from each unfolding curve. The values of *m* and $\Delta G_d(H_2O)$ were obtained directly by fitting the entire CD data set to Eq. (1). Table 4 summarizes the parameters obtained from chemical denaturation experiments. In comparison to the wild type, the $[GdnHCl]_{50\%}$ values, the concentration of guanidine hydrochloride required to denature 50% of the protein, of the mutants were increased. Moreover, positive values of $\Delta\Delta G_d(H_2O)$ for all of the variants (except the cXl mutant) obtained from chemical denaturation experiments supported the conclusion from thermodynamic study that all of the mutants have gained increased stability with respect to the wild type. Only the cXl mutant with $\Delta\Delta G_d(H_2O)$ value of −0.91 kcal/mol (Table 4) showed a reduction in the stability in comparison to the wild type xylanase. Clearly, because of the reduced slope of $\Delta G$ as a function of denaturant concentration, *m*, the estimate of $\Delta G_d(H_2O)$ of cXl mutant was much too low. This can be due to the existence of stable intermediate(s) in the unfolding transition of the cXl mutant that leads to breakdown of a two state assumption [24]. The presence of stable, partially unfolded intermediates at equilibrium would generally lower the *m* value compared to the value expected for a two state mechanism [20]. In order to minimize the error resulting from extrapolation to zero concentration of the denaturant due to variation of *m* [25], $\Delta\Delta G$ is best determined directly in the overlapping transition regions of mutants [26,27]. Thus, for practical reasons, $\Delta\Delta G_d$ was determined in 2.2 M GdnHCl concentration where transitions of all variants overlap and no extrapolation is required to obtain $\Delta\Delta G_d$ values. Similar to the thermal denaturation results, the DS1 mutant showed higher stability against chemical denaturation in comparison to the DS2 and cXl variants.

## 4. Discussion

The inherent instability of proteins seems to be a major obstacle in the application of enzymes in industry [5]. This limitation also applies to *B. circulans* xylanase as a potential candidate for bleaching Kraft pulp. It is of considerable interest, therefore, to increase its stability by modifying a few amino acids in the primary sequence. This appears to be possible as the net free energy of stabilization of native proteins is unexpectedly small.

It has been shown that removing naturally occurring disulfide bonds can substantially decrease the stability of proteins [28,29]. Consequently, the opposite process, the introduction of new disulfide bonds, should provide a powerful tool for protein stabilization. For example, introduction of three disulfide bonds into $T_4$ lysozyme has led to 23.4 °C increase in the melting point of the protein [30]. Thus, in the present work disulfide bonds were introduced into *B. circulans* xylanase in order to enhance its thermal as well as conformational stability. Circular dichroism spectra demonstrated that all of the mutants possessed the same conformation as the wild type protein (Fig. 2). Only the DS2 mutant showed a somewhat altered tertiary structure. Presumably, introduction of the disulfide bonds, except in the DS2 variant, was not accompanied by induction of any considerable strain in the structure of protein. This is a particularly important factor in designing disulfide bonds, as introduction of a strained bond can offset the stabilizing effect of the cross-link [5]. The lack of structural change, monitored by far- and near-UV CD, is consistent with an X-ray crystallographic study of the DS1 mutant showing that its structure is completely isomorphous with wild type enzyme [15]. Thus, it seems that 100C–148C mutations in DS1 mutant duly satisfy the geometric and stereochemical requirements for a disulfide bond formation. On the other hand, tertiary structure of the DS2 mutant was affected by the V98C,A152C mutations (Fig. 2). It is, therefore, anticipated that compared to DS1, these mutations should render not as much stability to the DS2 protein, because some of the stabilizing effects are offset by the strain introduced into the structure. This notion is supported by $\Delta\Delta G$ values obtained from thermal and guanidine hydrochloride denaturation experiments (Tables 3 and 4). Nonetheless, all of the mutants have acquired considerable stability against both chemical and thermal denaturation with the order of cDS1 > DS1 > cXl > DS2 > WT.

Often a combination of single point mutations leads to an additive increase in stability, i.e. the sum of the free energy changes derived from single mutations is nearly equal to the free energy change measured in the multiple mutant [31]. The cumulative nature of this phenomenon indicates that the structural stability effects of amino acid replacements are strictly

local. On the other hand, non-cumulative effects imply that there are some interactions between mutational sites, either through direct contact or indirectly through electrostatic interactions or structural perturbation [31,32]. In such cases, a combination of substitutions results in either a more or less stable protein, than if each substitution were to act independently. The term "cooperative stabilization" is used when stability achieved by a combination of mutations is more than expected. Mathematically, it can be expressed as $\Delta\Delta G_{coop} = \Delta\Delta G - \sum \Delta\Delta G_i$ [33]. The energy of cooperativity, $\Delta\Delta G_{coop}$, can be defined as the difference between the actual $\Delta\Delta G$ and the sum of the $\Delta\Delta G$ values of the individual changes, $\sum \Delta\Delta G_i$. $\Delta\Delta G$ values of various mutants obtained from differential scanning calorimetry experiments are listed in Table 3. The cDS1 variant has a $\Delta\Delta G$ value of 5.4 kcal/mol, which shows stability higher than sum of $\Delta\Delta G$ values (4.6 kcal/mol) for the combination of DS1 and cXl mutations. The difference, 0.8 kcal/mol, represents the energy of cooperativity, $\Delta\Delta G_{coop}$, in 2.5 M urea. The energy of cooperativity can also be calculated from guanidine hydrochloride denaturation experiments. As listed in Table 4, $\Delta\Delta G_d$ in 2.2 M GdnHCl for the cDS1 mutant equals 5.0 kcal/mol, which is 1.6 kcal/mol more than the sum of $\Delta\Delta G$ values (3.4 kcal/mol) of the individual single disulfide bond mutants. These data provide further proof that the combination of two disulfide bonds cooperatively stabilizes *B. circulans* xylanase. The cooperative stabilization of two disulfide bonds does not come as a surprise because a large loop formed by the A1GC,G187,C188 mutations in cXl variant is divided into two smaller loops by S100C,N148C mutations. Naturally, the smaller loops would restrict the backbone movement of the unfolded cDS1 mutant more than two independently acting disulfide bonds.

Polymer theory predict that a cross-link should stabilize a polymer according to the length of the primary chain bridged by the link due to a decrease in the chain entropy of the unfolded state of the cross-linked form [13]. If this assumption holds true, we should then expect higher stability for the cXl mutant in comparison to DS1, because the number of residues encompassed by the cross-link in the cXl mutant is much greater than DS1, 188 *versus* 49 residues (Table 3). It was found, from differential scanning calorimetry under reversible conditions, that DS1 with $\Delta\Delta G$ of 3.0 kcal/mol is more stable than the cXl mutant with $\Delta\Delta G$ of 1.6 kcal/mol. Since no structural change can be observed in the circular dichroism spectrum of cXl mutant compared to the wild type enzyme and no alteration in the specific activity of this mutant compared to the wild type protein, the lower thermal stability of the cXl mutant in comparison to the DS1 cannot be attributed to any considerable strain induced by the disulfide bond. Perhaps, it can be suggested that, after a certain point, increase in the number of residues forming a loop does not lead to enhanced stability. The $\Delta\Delta G$ values predicted by polymer theory using the equation suggested by Pace et al. [13] overestimated the free energy of stabilization for all of the mutants. As expected, the closest theoretical $\Delta\Delta G$ to experimental value was observed for DS1 mutant, which was still off by 1.5 kcal/mol. We do not know the exact reason behind this discrepancy but assume that, at least in part, it results from the assumption of volume of the sphere occupied by two –SH groups. It may also result from unwanted interactions of the folded state for some of the mutants. Taken together, the cooperative stabilization obtained by the combination of two disulfide bonds in cDS1 mutant should be attributed to the effect of disulfide bonds in the unfolded protein. This is consistent with the fact that the calorimetric enthalpy under reversible condition (Table 3) showed no major differences among mutants, while their $\Delta\Delta G$ values were found to be very different. Thus, the stabilizing effect of the disulfide bonds appears to be entropic [5,10,34].

We have previously shown that a comparison of the scan rate dependence of the melting points under irreversible conditions to those under reversible conditions can be used as a criterion to determine if the disulfide bond alters the rate limiting step on the thermal denaturation pathway [19]. Scan rate dependence of the melting points under reversible and irreversible conditions (in the presence and absence of urea) remained similar for the wild type, while in the case of DS1 mutant, the scan rate dependence under the reversible conditions was found to be much less than that for the irreversible one (Table 2). Therefore, it was concluded that the introduction of the S100C/N148C disulfide bond changes the rate-limiting step in thermal denaturation pathway probably by partially blocking the exposure of putative elements required for aggregation [19]. A similar strategy was used to determine the effect of introduction of disulfide bonds at various locations in the thermal denaturation pathway of the proteins. Accordingly, the melting point, $t_m$, of each mutant was determined in the presence and absence of 2.5 M concentration of urea at two scanning rates (Table 2). Since, the location of the disulfide bond in the DS2 mutant, V98C/A152C, was similar to that in DS1 (with the S100C/N148C mutation) a similar effect of disulfide bond on the rate-limiting step of thermal denaturation pathway was expected. This was indeed the case as dependence of the melting point of the DS2 mutant upon scanning rate under reversible conditions was less than for the irreversible case. A similar observation was made for the cDS1 variant, which consists of S100C/N148C, A1GC/G187,C188 mutations. On the other hand, the A1GC/G187,C188 disulfide bond did not change the rate limiting step indicating that the effect of disulfide bonds on the rate-limiting step of the thermal denaturation pathway was dependent on the location of the cross-link. These findings further support the notion that the presence of disulfide bonds in the DS1 and DS2 mutants, linking α-helix to the β-strand, restricts the exposure of hydrophobic surface and putative elements required for the initiation of aggregation. Taken together, the results support the attractive hypothesis [3] that cross-linking may provide a general means by which some conformational modes of inactivation may be blocked.

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