Terminal Amino Acids Disturb Xylanase Thermostability and Activity*§

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Background: Unlike the α-helix/β-strand, the non-regular region contains amino acid defined as disordered residue (DR); its effect on enzyme structure and function is elusive.

Results: Terminal DR deletions significantly increased xylanase thermostability and activity.

Conclusion: Terminal DRs disturb xylanase thermostability and activity.

Significance: DR deletion increased regular secondary structural content, and hence, led to slow decreased ΔG° in the thermal denaturation process, and ultimately, enhanced enzyme thermostability.

Protein structure is composed of regular secondary structural elements (α-helix and β-strand) and non-regular region. Unlike the helix and strand, the non-regular region consists of an amino acid defined as a disordered residue (DR). When compared with the effect of the helix and strand, the effect of the DR on enzyme structure and function is elusive. An Aspergillus niger GH10 xylanase (Xyn) was selected as a model molecule of (β/α)8 because the general structure consists of ~10% enzymes. The Xyn has five N-terminal DRs and one C-terminal DR, respectively, which were deleted to construct three mutants, XynΔN, XynΔC, and XynΔNC. Each mutant was ~2-, 3-, or 4-fold more thermostable and 7-, 4-, or 4-fold more active than the Xyn. The N-terminal deletion decreased the xylanase temperature optimum for activity (T_{opt}) 6 °C, but the C-terminal deletion increased its T_{opt} 6 °C. The N- and C-terminal deletions had opposing effects on the enzyme T_{opt} but had additive effects on its thermostability. The five N-terminal DR deletions had more effect on the enzyme kinetics but less effect on its thermostability than the one C-terminal DR deletion. CD data showed that the terminal DR deletions increased regular secondary structural contents, and hence, led to slow decreased Gibbs free energy changes (ΔG°) in the thermal denaturation process, which ultimately enhanced enzyme thermostabilities.

With enzyme thermostability. For example, thermophilic enzymes have stabilized secondary structures, such as increased residues within the strand, stabilized helix, and increased content of regular structure (1, 2). Helix and strand make stabilizing contributions (3, 4), but long loop make destabilizing contributions to protein thermostability (5). The stabilities of the helix and strand are mainly influenced by their N- and C-terminal residues (6–9). When compared with the effects of regular secondary structural elements, the effect of DR on enzyme structure and function is elusive. Additionally, because new N and C termini are often added or deleted to facilitate protein purification (10, 11), more information should be known about DRs.

An Aspergillus niger xylanase (Xyn) (Swiss-Prot number: A2QFV7) was selected as a model molecule of GH10 hydrolase because it plays an important role in biomass conversion and renewable energy production. The Xyn exhibits (β/α)_8 structure, and the general fold consists of ~10% enzymes, including amylase, glycosidase, triosephosphate isomerase, etc. (12). Based on sequence alignment with the mature Penicillium simplicissimum xylanase (Xyn-ps) and its structure (Protein Data Bank (PDB) code: 1BG4) (13), the Xyn is found to have five DRs at the N terminus (Gln-1–Ser-5) and one DR at the C terminus (Leu-302) that do not construct helix or strand (see Fig. 1). According to the negative correlation between non-regular content and the xylanase optimum temperature of activity (T_{opt}) (14), the DRs are assumed to disturb xylanase thermostability. To demonstrate the assumption, the five N-terminal DRs, the one C-terminal DR, and the six bi-terminal DRs, respectively, are deleted to construct three mutants, XynΔN, XynΔC, and XynΔNC. The DR deletions increase xylanase thermostability and catalytic efficiency, providing a new rational engineering method because rational engineering of enzyme thermostability and activity is still a great challenge (15–17).

EXPERIMENTAL PROCEDURES
Bacterial Strains and Reagents—According to the A. niger genome annotation (18), we cloned the Xyn gene into
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pET20b(+) (Novagen, Shanghai, China). The Xyn has 302 residues (SwissProt number: A2QFV7), consisting of the residue length of mature Xyn_ps (SwissProt number: P56588) (13). According to the Xyn_ps structure (1BG4) and sequence alignment, the Xyn has five N-terminal DRs (Gln-1, Ala-2, Ser-3, Val-4, and Ser-5) and one C-terminal DR (Leu-302) (see Fig. 1). The five N-terminal DRs, the one C-terminal DR, and the six bi-terminal DRs, respectively, were deleted to construct three mutants, Xyn\_N, Xyn\_C, and Xyn\_NC. Molecular reagents were Pfu polymerase, restriction endonucleases (NdeI/XhoI), T4 DNA ligase, DNA and protein marker, etc. (Takara Inc., Dalian, China).

Construction of the Mutants—The pET20b(+)-xyn served as template for the construction of deletion mutants (see Fig. 1). The genes xyn, xyn\_N, xyn\_C, and xyn\_NC were amplified by using the primers p1/p2, p3/p2, p1/p4, and p3/p4, respectively, with underlined letters showing Ndel and XhoI restriction sites. The sequences used are as follows: p1, ggaatccatatccg-acgtctcgtgatatga; p2, ccaaatctaactcgaggagacatttgccgccctgcag; p3, ggaatccatatccgtgatatcactaatggc; p4, ccaaatctaactcgaggagacatttgccgccctgcag. PCR was carried out by using 3.0 μl of pET20b-xyn as template, 1.0 μl of each of the related primers, 0.5 μl of Pfu polymerase, 4.0 μl of dNTPs, and 1× buffer. PCR conditions were: 4 min at 94 °C, 30 cycles (1 min at 94 °C; 1 min at 47 °C; 1 min at 72 °C), 10 min at 72 °C.

The amplified genes were cloned into pET20b(+) and digested with Ndel and XhoI to delete redundant restriction endonuclease sites (supplemental Fig. 1). After transforming Escherichia coli BL21(DE3)-competent cells, the recombinant plasmids were extracted and sequenced to confirm gene accuracy by using an ABI 3730 automated DNA sequencer (Invitrogen Biotechnology, Shanghai, China). The accurate transformants containing pET20b-xyn were screened on LB plates with ampicillin and X-gal (1.0 mM). The transformants were inoculated into 50 ml liquid LB medium containing ampicillin and X-gal (1.0 mM), and the media were shaken at 37 °C to log phase. Cells were centrifuged and resuspended in 5 ml of 1× PBS. Then, 1 ml of the resuspended cells were used to inoculate 200 ml of 1× PBS containing IPTG (0.4 mM) and ampicillin and X-gal (1.0 mM). The media were shaken at 37 °C to log phase and then induced by IPTG (0.4 mM). After induction at 25 °C, the cells were harvested by centrifugation and resuspended in 50 ml of 1× PBS for each transformation. The recombinant plasmids were extracted recombinant plasmids, the transformants containing pET20b-xyn were used for reported CD spectra, which were recorded from 195 to 260 nm using a 0.1-cm path-length cuvette at a scan speed of 50 nm/min, response time of 2 s, bandwidth of 2 nm, and pitch of 0.2 nm. For thermodynamic analysis, the thermal denaturation CD spectra were recorded at 220 nm from 30 to 80 °C upon heating at a constant rate of 2 °C/min. The molar ellipticities were calculated according to the following equation

\[ [\theta] = \frac{\theta \times MW}{100 \times l \times c} \]

where \([\theta], \theta, l, \text{ and } C\) are the molar and observed ellipticity, path length (in centimeters), and molar protein concentration, respectively. Thermal denaturation curves were fitted to a modified van’t Hoff equation using a reversible two-state protein unfolding model (20). The spectra data were fitted to the native and denatured transition baselines to obtain thermal melting temperature (T_m), enthalpy change (ΔH^o), entropy change (ΔS^o), and Gibbs' free energy change (ΔG^o) values (Sigmaplot, Version 10.0).

RESULTS

Construction of the Deletion Mutants—The DR-deleted genes produced specific DNA bands at ~1.3 kb on gel electrophoresis (Fig. 1). After we confirmed the accuracies of the extracted recombinant plasmids, the transformants containing pET20b-xyn\_N, pET20b-xyn\_C, and pET20b-xyn\_NC were grown and induced to extract xylanases. The enzymes Xyn\_N, Xyn\_C, and Xyn\_NC produce specific protein bands at ~35 kDa on SDS-PAGE (Fig. 1, Table 1). The larger apparent molecular masses are attributed to the xylanases having acidic properties and a His_N tag at the C termini, because acidic protein was found to bind less SDS and therefore had a larger apparent molecular mass (21–23).

Enzyme Property—The T_{opt}, values of Xyn\_N, Xyn\_C, and Xyn\_NC are 38, 50, and 44 °C, respectively, which are ~6 °C lower, 6 °C higher, and equivalent to that of Xyn (Fig. 2, Table 1). Thereby, the N-terminal deletion decreased, but the C-terminal deletion increased xylanase T_{opt} 6 °C, whereas the N- and C-terminal deletions had an opposing effect on the xylanase T_{opt}. The three mutants had equivalent pH_{opt} values to the Xyn (Table 1, supplemental Fig. 2), indicating that the DR deletions did not alter the xylanase pH property.

After incubation at 50 °C, the t_{1/2} values of Xyn\_N, Xyn\_C, and Xyn\_NC are 54.5, 74.7, and 114.3 min, respectively, which are ~2–3, or 4-fold longer than that of Xyn (Fig. 2). The N- and C-terminal deletions had an additive effect on xylanase thermostability because the half-life of Xyn\_NC is approximately the sum of those of Xyn\_N and Xyn\_C. However, the one C-terminal DR had more of an effect on xylanase thermostability than the five N-terminal DRs.

After kinetic analysis, xylanase activities increased in the order of Xyn\_NC < Xyn\_C < Xyn\_N. Each V_{max} is ~3.8-,
4.1-, or 7.3-fold higher than the Xyn (Table 1, supplemental Fig. 3). The N- and C-terminal deletions had no additive effect, but had a slight opposing effect on xylanase activity. The three mutants increased catalytic activity for xylan, but decreased affinity for xylan. Thus, the more that substrates bind to an enzyme, the higher its activity. The five N-terminal DR deletions had more of an effect on xylanase activity and its affinity than that of the one C-terminal DR deletion. The

![Construction of deletion mutants](image1)

**FIGURE 1.** Construction of deletion mutants. Upper, helix, strand, and non-regular region are shown in as boxes, arrows, and lines, respectively. The five N-terminal DRs are Gln-1, Ala-2, Ser-3, Val-4, and Ser-5, and the one C-terminal DR is Leu-302. Lower, the xylanases Xyn, XynΔC, XynΔN, and XynΔNC were amplified by using the primers p1/p2, p1/p4, p3/p2, and p3/p4, respectively. The three mutants produce specific DNA bands at ~1.3 kb on gel electrophoresis (left) and specific protein bands at ~35 kDa on SDS-PAGE (right). The M lane indicates molecular mass markers.

| Number of residues | Molecular mass (theoretical vs. apparent) | T<sub>ω</sub> | ΔS<sup>b</sup> | ΔC<sup>b</sup> | ΔH<sup>ω</sup> × 10<sup>3</sup> | T<sub>opt</sub> | V<sub>max</sub> μmol·liter<sup>−1</sup>·min<sup>−1</sup> vs. K<sub>m</sub>(mg/ml)<sup>d</sup> | t<sub>1/2</sub> <sup>e</sup> |
|-------------------|-------------------------------------------|----------|-------------|-------------|------------------|--------|-----------------------------------------------|--------|
| Xyn               | 312                                       | 34.1/37.56 | 50.5        | 4415.9      | 1225.4           | 44     | 659.6/19.7                                   | 28.7   |
| XynΔN             | 307                                       | 33.6/35.79 | 46.4        | 1456.6      | 1428.8           | 44     | 4860.8/46.5                                 | 54.5   |
| XynΔC             | 311                                       | 34.0/37.04 | 50.5        | 2618.2      | 464.5            | 44     | 2403.8/27.4                                 | 74.7   |
| XynΔNC            | 306                                       | 33.6/35.55 | 50.8        | 2173.5      | 703.8            | 44     | 2503.8/26.5                                 | 114.3  |

<sup>a</sup> T<sub>ω</sub>, the heat melting temperature.

<sup>b</sup> H<sup>ω</sup>, ΔS<sup>b</sup>, and ΔC<sup>b</sup>, the molar enthalpy change, entropy change, and heat capacity change between native and denatured states of enzyme.

<sup>c</sup> T<sub>opt</sub>, optimal temperature for activity.

<sup>d</sup> K<sub>m</sub> and V<sub>max</sub> were calculated according to kinetic analysis by reaction at each enzyme optimal condition.

<sup>e</sup> t<sub>1/2</sub>, the thermal denaturation half-life, which was assayed for residual activity after incubation at 50 °C for a 10-min interval from 0 to 100 min and was compared with the unincubated enzyme.

![Optimum activity temperatures and thermostabilities of xylanases](image2)

**FIGURE 2.** Optimum activity temperatures (left) and thermostabilities (right) of xylanases. Residual activities were assayed for the xylanases after incubation at 50 °C for a 10-min interval from 0 to 100 min.
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FIGURE 3. Unfolded fractions (left) and molar Gibbs free energy changes (ΔG°) (right) in thermal denaturation process of xylanases. The denaturation CD data were measured at 222 nm from 303 to 336 K upon heating at a rate of 2 °C/min and were converted to an unfolded fraction using the equation (fe = (y − yd)/(yd − yf)), where y is the ellipticity observed at the given temperature and yd and yf are the characteristic ellipticities of the folded and unfolded protein. The fe spreads to the vicinity of pre- and post-transition baselines at low and high temperatures. ΔG°(dG, k/mol) was calculated according to the standard van’t Hoff equation (ΔG° = −RT(Δf/1 − f)) using a two-state transition model.

affinity of XynΔNC was higher than those of XynΔN and XynΔC.

CD Analysis—According to the thermal denaturation CD spectra, the Tm values of XynΔN, XynΔC, and XynΔNC are 46.4, 50.5, and 50.8 °C, respectively, which are 4.1 °C lower than, equivalent to, and 0.3 °C higher than that of Xyn, respectively (Table 1, Fig. 3). Because the mutant Tm values were not consistent with their Tg values, the enhanced enzyme thermostability was not caused by the increase of Tm. After calculating the enzymatic ΔG° values between the native and denatured states, we determined that the slopes of ΔG° profiles decrease slowly in the order of Xyn < XynΔC < XynΔN < XynΔNC (Fig. 3). Thus, the enhanced thermostabilities consist of the slow decreased ΔG° values, and this agrees with thermodynamic analysis of the two variants of Streptomyces halstedii xylanase (24). Similar to the ΔG° data, the xylanases unfold quickly in the order of Xyn > XynΔC > XynΔN > XynΔNC (Fig. 3). Because its Tm is lower, the XynΔN is less thermostable than the XynΔC. The XynΔNC is the most thermostable enzyme because it has the highest Tm and the slowest decreased ΔG°. The calculated heat capacity change (ΔC) values show decreasing order of Xyn > XynΔC > XynΔN > XynΔNC (Table 1). Thus, the decreased ΔC tendency is also comparable with the enhanced thermostability. The tendency is also shown in the thermodynamic analysis of the ribosomal protein L30e and the Bacillus subtilis xylanase (11, 25). However, the other two thermodynamic parameters (ΔS and ΔH°) have no general tendencies in thermal denaturation process.

To compare whether or not regular secondary structural contents increased after the DR deletions, the xylanase CD data were used to dissect secondary structural elements because α-helix, β-sheet, and irregular coil have characterized negative CD spectrum bands at ~222 and 210 nm, ~215 nm, and ~195 nm, respectively. All of the CD profiles have two clear minima at 210 and 222 nm (Fig. 4), agreeing with GH10 xylanase having both α-helix and β-sheet features. The three mutant CD spectra retain unchanged minima at 222 nm, although the relative amplitudes decreased when compared with that of the Xyn. Because irregular coil has a characterized negative band at ~195 nm, the spectra minima at 210 nm shift toward higher wavelengths in the order of Xyn < XynΔN < XynΔNC < XynΔC, indicating that the contents of helix and strand within the mutants increased relatively after the DR deletions when compared with that of the Xyn. Thus, the terminal DRs disturb enzyme stability, indicated by the decreased GB1 stability after elongation of a loop (5).

Structural Analysis—To analyze the terminal DR effects from a structural level, the Xyn structure was modeled (Fig. 4) based on the Xyn_ps structure (1B31). According to sequence alignment with the Thermotoga maritima xylanase B and its structure annotation (1VBR) (26), we found the active-site residues (Glu-132, Glu-238) and substrate-binding sites (Lys-51, Arg-82, Trp-88, Gln-208, and Trp-268) of the A. niger Xyn. The five N-terminal DRs and the one C-terminal DR do not construct the helix or strand of the (β/α)s structure, and the new N and C termini become closer to each other after the deletions, which makes the structure become more compact. Moreover, the disulfide bond Cys-256–Cys-262 probably becomes tighter after the DR deletions. As to the effect on catalytic activity, the N-terminal DRs (Gln-1–Ser-5) and the helix containing the C-terminal DR (Leu-302) are close to the residue Trp-276, which was found to form a “lid” to partially shield the active-site residues (Glu-132 and Glu-238) in the P. simplicissimum xylanase (13). After the DR deletions, substrate probably enters more easily to the substrate-binding sites, Lys-51, Arg-82, Trp-88, Gln-208, and Trp-268 of the A. niger Xyn (Fig. 4). Because a cluster of N- and C-terminal residues is involved in substrate binding, the C-terminal deletion changed the substrate binding specificity of the phospholipase A2 (27). Similarly, deletion of the 10-kDa C-terminal peptide caused steric hindrance of the substrate toward the catalytic concave cleft of Fibrobacter succinogenes 1,3-1,4-β-D-glucanase (28).
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FIGURE 4. CD spectra (left) and structure (right) of xylanase. The CD spectra (left) were determined from 195 to 260 nm. Protein concentrations were 0.699, 0.789, 0.697, and 0.777 mg/ml for the xylanases, Xyn, XynΔC, XynΔN, and XynΔNC, respectively. Similar to the Xyn, the three mutants exhibit broad CD spectra bands with minima at 210 and 222 nm, consisting of (β/α)2 structure of GH10 xylanase having compact features of α-helix and β-sheet. Thereby, the three deletion mutants fold properly at room temperature. Because irregular coil has a characterized negative band at 195 nm, the shifts of minima at 210 nm toward higher wavelength show that the irregular coil contents of the xylanase decrease after the DR deletions, and therefore, the contents of strand and helix increase relatively in the order of Xyn < XynΔN < XynΔNC < XynΔC, according to CD reference spectra by Dr. J. T. Yang (JASCO software package). The A. niger Xyn structure (right) was modeled by using the Swiss-model software (39, 40), with the β-sheet shown with an arrow, the α-helix shown with spiral ribbon, and the irregular coil shown as a line. According to sequence alignment with the T. maritima xylanase B and its structure (1VBR) (26), we found active-site residues (Glu-132, proton donor, and Glu-238, nucleophile) and substrate-binding sites of the A. niger Xyn. The N-terminal DRs (Gln-1, Ala-2, Ser-3, Val-4, and Ser-5) and the helix containing the C-terminal DR (Leu-302) are close to the Trp-276 (13), which was found to form a lid to partially shield the active-site residues (Glu-132 and Glu-238). After the DR deletions, substrate enters more easily into the substrate-binding sites (Lys-51, Arg-82, Trp-88, Gln-208, and Trp-268). Additionally, new N and C termini become closer to each other after DR deletions, which makes the Xyn structure more compact, and therefore, increases its thermostability.

DISCUSSION

Previously, either the entire domain of the enzyme was truncated completely, or terminal residues of the enzyme were successively deleted (27–33). The present study deleted the six terminal DRs based on structural analysis, respectively, and the deletions significantly increased the xylanase thermostability and activity. Similar to our data, the N-glycanase increased deglycosylation activity after deletion of the N-terminal helix (29). After successive truncation analysis, the seven N-terminal residues and the three C-terminal residues were shown to be unnecessary for the Clostridium thermocellum lichenase catalytic activity (30). As to enzyme thermostability, the successive deletion of terminal residues decreased the phosphoribosyltransferase thermostability (31); probably, its structure and conformation were disturbed by the successive deletions. Similar to our data, the extra four N-terminal residues decreased the lysozyme thermostability and its refolding rate (10). Deletion of the four C-terminal glycine-rich repeats and the domain increased enzyme thermostability, substrate binding affinity, and activity, respectively (28, 30, 32). Residue mutations happened generally in the N termini of the 15 most thermostable xylanases, showing that the N-terminal region was more susceptible to thermal unfolding (9). The five N-terminal mutations might confer structural stability, and hence, prevent the overall thermal unfolding of the mesophilic Streptomyces olivaceoviridis xylanase (34). The N and C termini were found to play very prominent roles in the inactive and active fold switch of the B. subtilis xylanase (11). Although commonly used in facilitating protein purification by adding or deleting N- or C-terminal residues, newly created residues interfered with protein structure and function (11). Thus, in functional analysis, the protein should be complete and have no extra residues, as well as being free of unnecessary residues encoded by redundant endonuclease sites in expression vectors whenever possible.

The parameters, the T_{\text{opt}}, t_{1/2}, and T_m were assayed to describe the different thermal features of the xylanases. T_{\text{opt}} reflects enzymatic activity at a certain temperature. An enzyme with a higher T_{\text{opt}} is commonly regarded as having a higher thermostability (12), and it is often described by the organism growth temperature optimum. t_{1/2} reflects the thermal resistance of an enzyme at a certain temperature; therefore, it is more suitable to indicate enzyme thermostability. When compared with the Xyn, the XynΔN had a lower T_{\text{opt}} but a longer t_{1/2}. The difference indicates that the two parameters are not always consistent, especially for mutant and wild type of a same enzyme, and that the two parameters connect with different structural features. Unlike the two previous parameters, the T_m values were assayed to show 50% of the xylanases being thermally inactivated after continuous incubation from 30 to 80 °C upon heating at a rate of 2 °C/min; therefore, the T_m values of three mutants are higher than their T_{\text{opt}} values. Moreover, the Photinus pyralis (firefly) luciferase T_m reflects a thermal deactivation intermediate state that includes reversible active and inactive states (35).

Because the five N-terminal DRs (Gln-1–Ser-5) and the one C-terminal DR (Leu-302) do not form regular secondary structural elements, the DR deletions increased contents of the helix and strand within the enzyme, and thus, led to slow decreased ΔG° tendency in the thermal denaturation process. Thermophilic proteins had more stable helices and larger residue fractions within helical conformations than mesophilic counter-
parts (36, 37). The regular structural element has a stabilizing contribution to enzyme thermostability (14). The ΔGθ values reflect global conformational changes and consist of the decreased mechanical strength induced by thermal denaturation (25). After elongation of the second loop, the GB1 decreased mechanical strength by 64% for 46 residues (5). When the N terminus of the GB1 was extended from its C terminus, the structure was damaged more easily by external force (4). Robust proteins have more mechanical stabilities and higher stiffness, and hence, are less responsive to external perturbation (38).

In summary, the A. niger xylanase terminal DRs were deleted based on the structural analysis. The deletions enhanced the mesophilic xylanase thermostability by 2–4-fold and increased its catalytic activity by 3.8–7.3-fold more than those of the wild type. The five N-terminal DR deletions decreased the xylanase T°opt by 6 °C, but the one C-terminal DR deletion increased its T°opt by 6 °C, respectively. Thus, the N- and C-terminal DR deletions had opposing effects on the enzyme T°opt, but had additive effects on its thermostability. CD analysis showed that the DR deletions increased the contents of the helix and strand within the enzyme, and hence, led to slow decreased ΔGθ in the protein thermal denaturation process, and ultimately, enhanced the xylanase thermostability.

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