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ORIGINAL PAPER

Introduction of a disulfide bridge enhances the thermostability of a *Streptomyces olivaceoviridis* xylanase mutant

H. M. Yang · B. Yao · K. Meng · Y. R. Wang · Y. G. Bai · N. F. Wu

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Abstract Substitution of the N-terminus of *Streptomyces olivaceoviridis* xylanase XYNB to generate mutant TB has been previously shown to increase the thermostability of the enzyme. To further improve the stability of this mutant, we introduced a disulfide bridge (C109–C153) into the TB mutant, generating TS. To assess the effect of the disulfide bridge in the wild-type enzyme, the S109C-N153C mutation was also introduced into XYNB, resulting in XS. The mutants were expressed in *Pichia pastoris*, the recombinant enzymes were purified, and the effect of temperature and pH on enzymatic activity was characterized. Introduction of the disulfide bridge (C109–C153) into XYNB (XS variant) and TB (TS variant) increased the thermostability up to 2.8-fold and 12.4-fold, respectively, relative to XYNB, after incubation at 70°C, pH 6.0, for 20 min. In addition, a synergistic effect of the disulfide bridge and the N-terminus replacement was observed, which extended the half-life of XYNB from 3 to 150 min. Moreover, XS and TS displayed better resistance to acidic conditions compared with the respective enzymes that did not contain a disulfide bridge.

Keywords Xylanase · Thermostability · Disulfide bridge · N-terminus replacement · Synergistic effect

H. M. Yang · B. Yao (✉) · K. Meng · Y. R. Wang · Y. G. Bai
Feed Research Institute,
Chinese Academy of Agricultural Sciences,
100081 Beijing, China
e-mail: yaobin@public3.bta.net.cn

N. F. Wu
Biotechnology Research Institute,
Chinese Academy of Agricultural Sciences,
100081 Beijing, China

Introduction

Xylanase (EC3.2.1.8) can hydrolyze xylans into xylo-oligosaccharides and D-xylose. Endo-β-D-1,4-xylanase, one of the major hemicellulose hydrolases, catalyzes the hydrolysis of internal β-1,4 bonds of xylan [20]. Currently, many endo-β-D-1,4-xylanases from different sources are primarily classified into the glycosyl hydrolase families, F/10 and G/11, according to their structural similarity [3, 16]. Although xylanases have been widely used, their application in the paper, pulp and feed industries is limited somewhat by certain enzymatic characteristics such as poor resistance to high temperature and narrow optimal pH range [24]. Therefore, it is essential to improve the properties of xylanases for broader applications.

Thermostability is defined as the ability of an enzyme to remain stable and active after storage at high temperature (e.g., 70°C) without substrate. This is affected by several complicated factors in xylanases, such as thermostability domains [2, 26], salt bridges, aromatic interactions [6, 10, 14, 15, 28], disulfide bridges [5, 9, 27, 29] and polar side chains of the protein surface. The methods of site-directed mutagenesis [11, 17, 30] and directed evolution [18, 19, 23] have been used to improve the thermostability of xylanases. Furthermore, replacement of the N-terminus of family G/11 xylanases with the corresponding region of *Thermomonospora fusca* xylanase TfxA improved the thermostability of some xylanases [12, 22, 25, 31, 33].

Streptomyces olivaceoviridis xylanase XYNB (GenBank accession number: AJ292317) has a molecular weight of 21 kD and had been predicted to contain two large β-pleated sheets and one α-helix region [33, 34]. This family G/11 xylanase is broadly used in the feed

industry by virtue of characteristics such as high specific activity and strong resistance to pepsin and trypsin, but its use is limited by its low thermostability. Consequently, to improve the thermostability of XYNB, the mutant TB (GenBank accession number: DQ465452) was constructed by replacing the N-terminus 33 residues with 31 residues of *T. fusca* xylanase TfxA in the corresponding region. TB had six-fold higher thermostability after incubation at 80 or 90°C for 3 min [33]. In the present study, we introduced one disulfide bridge into both the wild-type XYNB (to yield mutant XS) and TB (to yield mutant TS) and compared the enzymatic properties of these xylanases.

Materials and methods

Expression vector, genes and host strains

The yeast *P. pastoris* strain GS115 (*his4*) and the integrative expression vector pPIC9 were purchased from Invitrogen (Carlsbad, CA, USA). The wild-type gene *xynB* and the hybrid gene *tb* were cloned into pPIC9 using the *Eco*R I and *Not* I recognition sites, and the pPIC9 + *xynB* and pPIC9 + *tb* recombinant plasmids were obtained with the correct ORFs as determined by sequencing.

Construction of pPIC9 + *xs* and pPIC9 + *ts*

The structures of the xylanase variants were predicted by Swiss Model and Swiss-pdb Viewer (http://www.expasy.ch/spdbv/) [8], and mutations of S109C and N153C were presumed to form a disulfide bridge between the B9 β-sheet and the N-terminus of the α-helix region in XS and TS respectively.

The S109C and N153C mutations were inserted into *xynB* and *tb* using the oligonucleotide primers S109C, 5′-GGCACGGTCACCTGCGACGGCGGCACG-3′ (mutant nucleotide underlined), and N153C, 5′-ATC ACCACCGGCTGCCACTTCGACGCC-3′ (mutant nucleotides underlined), following the vitro Mutagenesis System protocol (Promega, Madison, WI, USA) and then subcloned into plasmid pPIC9 using the *EcoR* I and *Not* I recognition sites to generate pPIC9 + *xs* and pPIC9 + *ts*.

Expression and purification of XYNB, TB, XS and TS

Approximately 10–20 μg of the recombinant plasmids (pPIC9 + *xynB*, pPIC9 + *tb*, pPIC9 + *xs* and pPIC9 + *ts*) were linearized with *Bgl* II and then transformed into *P. pastoris* strain GS115 by electroporation according to the Invitrogen manual (http://www.invitrogen.com/content/sfs/manuals/pich_man.pdf). The transformants were fermented in BMGY culture medium (200 ml; 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (w/v), 4×10^{-5}% biotin (w/v), 1% glycerol (w/v)) at 30°C, shaking at 250 rpm for 48 h. After harvesting by centrifugation, the yeast cells were cultured in BMMY culture medium (50 ml; 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (w/v), 4×10^{-5}% biotin (w/v), 0.5% methanol (v/v)) in 500-ml flasks at 30°C, shaking at 250 rpm for 72 h, and 0.5% methanol (v/v) was added to this medium every 12 h to induce the secretion of xylanase. The yeast cells were removed by centrifugation at 6,000*g* for 15 min, and cell-free supernatants (2 ml) were loaded on a 120-ml bed volume Sephacryl-S200 column (Pharmacia, Sweden). Each enzyme was eluted with 50 mM citric acid-Na_2HPO_4 buffer (pH 6.0) at a flow rate of 1 ml/min, and purified with a 5 kD Nanosep Centrifugal Device (Pall Life Science, East Hills, NY, USA).

Enzyme analysis

Xylanase activity was analyzed using 1% birch wood xylan (w/v) as the substrate in the McIlvaine buffer system (0.2 M sodium phosphate, 0.1 M citric acid, pH 6.0), at 60°C for 10 min (the standard conditions for xylanase XYNB). The sugar released was quantified by the method of Somygi-Nelson [32]. One unit of xylanase activity was defined as the amount of enzyme that catalyzed the formation of 1.0 μmol of reducing sugar from xylan per minute under the conditions mentioned above, and each sample was measured in triplicate. The protein concentration was analyzed by the Bradford method with bovine serum albumin as the control [1].

The formation of disulfide bridges was demonstrated as previously described [29] by adding 1–5 mM dithiothreitol (DTT) in the presence of 1% SDS at 70°C for 5 min prior to SDS-PAGE. To evaluate whether the increase in thermostability resulted from the introduction of a disulfide bridge, the thermostability of the mutants at 70°C was assessed both in the presence and absence of DTT.

Enzyme properties

To determine the pH optimum, the xylanase activity was measured from pH 2.2 to 8.0 in 100 mM McIlvaine's buffer and at pH 9.0 in 100 mM Gly-NaOH buffer using 1% birch wood xylan as substrate, at 60°C for 10 min. The pH value at which xylanase has the highest activity is the pH optimum. To determine

the temperature optimum, the xylanase activity was measured from 30 to 90°C using 1% birch wood xylan as substrate, in 100 mM McIlvaine's buffer at pH 6.0 for 10 min. The temperature at which xylanase has the highest activity is the temperature optimum.

The stabilities of the xylanase variants at different pHs were determined by incubating the enzymes in different pH buffers at 60°C for 30 min in the absence of substrate. The control was put on ice and not subjected to preincubation. The activities of the pretreated enzymes and the controls were measured under the standard conditions described above under 'Enzyme analysis'. The residual activity was calculated relative to the 100% activity of the control. In addition, the thermostability of each xylanase variant was measured by incubating the enzyme for different periods at 70°C, pH 6.0. After chilling on ice, the activities of the pretreated enzymes and the controls were measured under the standard conditions. The residual activity and half-life were calculated for each xylanase. The control was put on ice and not subjected to preincubation.

To determine the K_m and V_{max}, the reaction duration was set at 10 min and the concentration of xylan ranged from 0.125 to 2.0%. The xylanase activity was measured under the standard conditions. The kinetic parameters were determined according to the Michaelis–Menten equation using double-reciprocal plots.

Results

Disulfide bridge in XS and TS mutants

The genes for XS and TS were cloned into the expression vector pPIC9 and confirmed to be correct by sequencing. Figure 1 presents the predicted structure of the mutants based on the coordinates of PDB entries 1hix.pdb, 1m4w.pdb, 1xnc.pdb, and 1te1.pdb (*streptomyces sp.*S38 xylanase XYL1; *nonomuraea flexuosa* xylanase; one glycosidase; one hydrolase). C109 in the B9 β-sheet is adjacent to C153 in the α-helix region, and the distance between the two thiol groups is 3.56 Å. Thus, a disulfide bridge can potentially form between the B9 β-sheet and the N-terminus of the α-helix region.

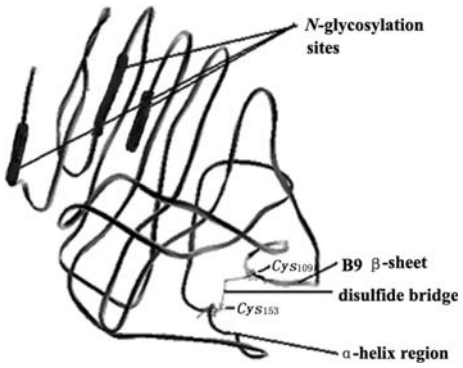

Fig. 1 Putative structure of xylanase variants containing a disulfide bridge. The structure was created based on the coordinates of PDB entries (1hix.pdb, 1m4w.pdb, 1xnc.pdb, and 1te1.pdb) using the Swiss-pdb Viewer and Swiss Model programs

Expression and purification of xylanases

The *P. pastoris* GS115 transformants were used for further fermentation. During high-density fermentation, the xylanases accumulated and the activities reached an average yield of 300 IU/ml in the supernatant of the culture medium after 72 h of induction. After purification through a Sephacryl-S200 column and concentration with the 5 kD Nanosep Centrifugal Device, the specific activities **(**IU/mg) of XYNB, TB, XS and TS were 886.89, 883.36, 773.46, and 523.33, respectively (Table 1). The XYNB, XS, TB and TS proteins expressed in *P. pastoris* all had the same molecular weight of 31 kD (Fig. 2)**.** We previously observed that N-glycosylation is responsible for the higher molecular weight of recombinant xylanases expressed in *P. pastoris.* The molecular weight of XYNB expressed in *P. pastoris* reverts to 21 kD after digestion with endoglycosidase [33]. There are three N-glycosylation sites in XYNB and XS (N-Q-T, N-Y-S, N-I-T). The first N-glycosylation site was part of the N-terminus that was substituted for the construction of TB, so N-glycosylation sites in TB and TS are N-E-T, N-Y-S and N-I-T (Fig. 1) [7].

Enzyme properties

No disulfide bridge can form in the wild-type xylanase XYNB as it contains only a single cysteine residue.

Table 1 Comparison of enzymatic properties of XYNB, XS, TB, and TS

Enzymatic properties[a]	XYNB	XS	TB	TS
Optimum temperature (°C)	60	65	70	70
Optimum pH	6.0	6.0	6.0	6.0
pH stability[b]	4–9	3–9	5–9	4–9
K_m (g/kg)[c]	20.87	29.90	28.75	33.60
V_{max} (μmol/mg • min)	4568	3333	9397	4200
Specific activity (IU/mg)	886.89	773.46	883.36	523.33
Half-life (min)	3	9	20	150

[a] Each enzymatic property was measured in triplicate

[b] pH range in which residual activity was above 50% after incubation at 60°C for 30 min

[c] Unknown molecular weight and concentration of xylan required us to use g/kg as the unit for K_m

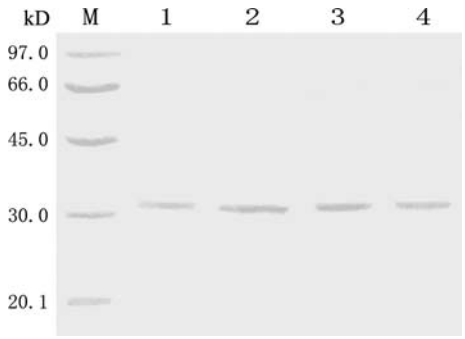

Fig. 2 SDS-PAGE analysis of the purified xylanases. SDS-PAGE was performed on 12% (w/v) polyacrylamide gel. Protein was stained with Coomassie Brilliant Blue R-250. Lanes: M, protein marker; 1, purified XYNB; 2, purified TB; 3, purified XS; 4, purified TS

However, altered mobility in SDS-PAGE after treatment with different DTT concentrations indicated that disulfide bridges were formed in the mutants XS and TS (Fig. 3).

After treatment with 10 mM DTT, at 4°C for 12 h, the thermostability of XS and TS was decreased by about two-fold at 70°C; the thermostability of DTT-treated XS was equivalent to that of DTT-treated XYNB**,** suggesting that DTT disrupted the disulfide bonds formed in XS. However, the thermostability of TS after treatment with DTT was still higher than that of DTT-treated TB, which might reflect the synergistic effect between the disulfide bridge and the N-terminus replacement (data not shown).

The optimum pH for the wild-type and mutants was 6.0 (Table 1); however, the pH at which the residual activity was ≥50% after incubation at 60°C for 30 min varied among the xylanases (Fig. 4). At pH 2 to 3, the residual activities for TS, XS, and XYNB were above 35%, whereas the residual activity was less than 10% for TB. At pH 4, the residual activity for XS was nearly 100%, but it was only 45% for TB. In addition, at pH 6–8, the residual activities of the four xylanases were very similar.

The optimum temperatures of the purified XYNB, XS, TB and TS at pH 6.0 were 60, 65, 70, and 70°C, respectively (Table 1). After incubation at 70°C, pH 6.0, for 20 min, TS retained 100% of the original activity whereas TB and XS retained 50 and 23%**,** respectively, and XYNB only retained 8% (Fig. 5). When the incubation duration was extended to 150 min, the residual activity of TS was 53%; however, the residual activity of TB was only 3% (data not shown), and

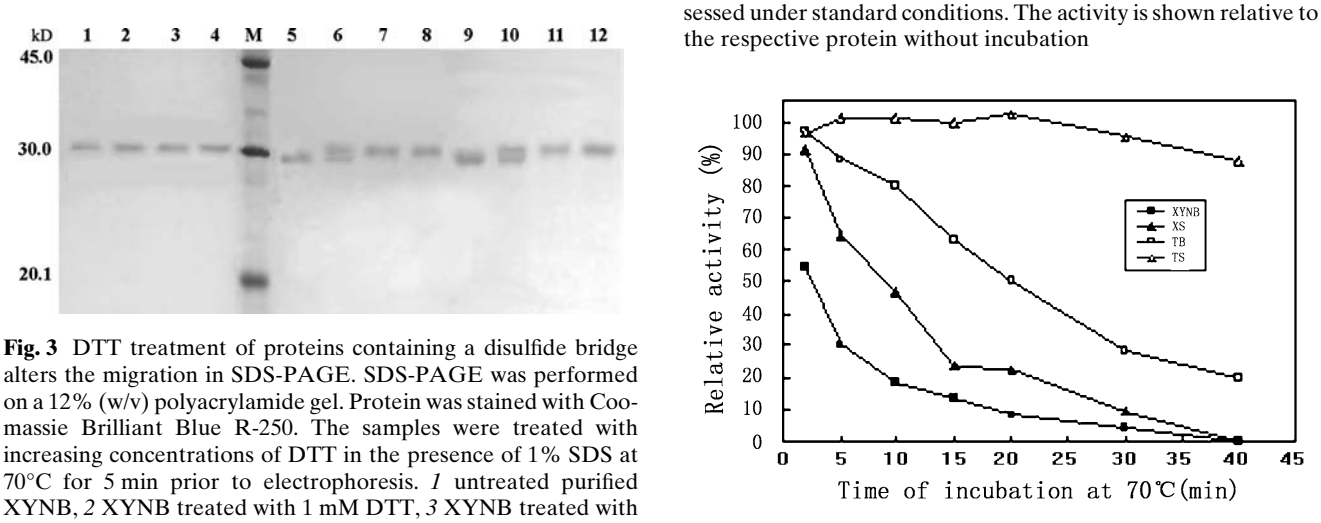

Fig. 3 DTT treatment of proteins containing a disulfide bridge alters the migration in SDS-PAGE. SDS-PAGE was performed on a 12% (w/v) polyacrylamide gel. Protein was stained with Coomassie Brilliant Blue R-250. The samples were treated with increasing concentrations of DTT in the presence of 1% SDS at 70°C for 5 min prior to electrophoresis. *1* untreated purified XYNB, *2* XYNB treated with 1 mM DTT, *3* XYNB treated with 2 mM DTT, *4* XYNB treated with 5 mM DTT, *M* low-molecular weight protein marker, *5* untreated purified XS, *6* XS treated with 1 mM DTT, *7* XS treated with 2 mM DTT, *8* XS treated with 5 mM DTT, *9* untreated purified TS, *10* TS treated with 1 mM DTT, *11* TS treated with 2 mM DTT, *12* TS treated with 5 mM DTT

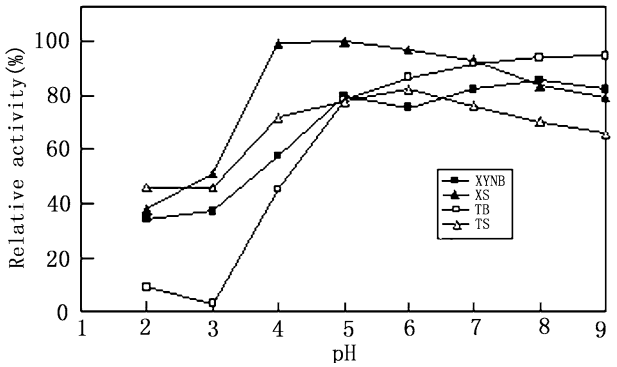

Fig. 4 Effect of pH on XYNB, XS, TB and TS activity. The purified proteins were incubated in buffer at the indicated pHs for 30 min at 60°C, and the residual activity was subsequently assessed under standard conditions. The activity is shown relative to the respective protein without incubation

Fig. 5 Effect of temperature on XYNB, XS, TB and TS activity. The purified proteins were incubated at 70°C for the indicated periods, cooled on ice, and the residual activity of the enzymes was subsequently assessed under standard conditions. The activity is shown relative to the respective protein without incubation at 70°C

XYNB and XS had no activity after incubation at 70°C for only 40 min (Fig. 5). The half-lives for TS, TB, XS, and XYNB were 150, 20, 9 and 3 min, respectively (Table 1).

The K_m and V_{max} values of XYNB, XS, TB, and TS are shown in Table 1. XS, TB and TS were as resistant to trypsin and pepsin as XYNB, and none of them showed cellulase activity (data not shown).

Discussion

The presence of disulfide bridges changes the mobility of the protein in SDS-PAGE. Enzymes that do not contain a disulfide bridge tend to bind more SDS, and thus, migrate slower than the enzymes with an intact disulfide bridge [29]. In the presence of 1 mM DTT, the XS and TS mutants displayed two bands in SDS-PAGE (Fig. 3, lanes 6 and 10). XS and TS migrated slower when the concentration of DTT was higher than 2 mM, due to reduction of the disulfide bond (Fig. 3, lanes 7, 8, 11, 12). However, the wild-type displayed no mobility difference in the presence of DTT (Fig. 3, lanes 2–4). The disulfide bridges in XS and TS were also identified using ESI-Q-TOF mass spectrometry (data not shown), thus supporting the electrophoretic data.

The N-terminal, C-terminal, and α-helix regions of xylanases are important factors in the thermostability due to their labile structures. Xylanase mutant TB was constructed by replacing the N-terminus of XYNB with the N-terminus of the most thermophilic and thermostable xylanase, TfxA [33], which improved the thermostability. Moreover, the thermostability of mutant TS (containing the N-terminus substitution and the disulfide bridge) was much higher than that of both mutant XS and mutant TB, which may reflect a potential synergistic effect between the disulfide bridge and the N-terminus replacement. These results may help to elucidate the mechanism responsible for thermostability.

Although the disulfide bridge is one of the factors essential for improving the thermostability of xylanases, other factors cannot be neglected [21]. For example, the half-life of *Trichoderma reesei* xylanase XYN II mutant (containing an engineered disulfide bridge) was increased from 1 to 14 min at 65°C, and up to about 100 min after adding mutations Q162H/Q162Y, N11D and N38E [27]. Furthermore, another disulfide bridge introduced between residues 2 and 28 in the above mutant improved the thermostability by 5,000-fold, and its half-life reached 25 min even at a high temperature of 75°C [5, 9]. Similarly, in the present study, the combination of disulfide bridges and N-terminus replacement (containing mutations Q8E, T11Y, N12H, N13D, G24P, T30E and S33P) [33] might be more effective in improving the thermostability than a single mutation.

The wild-type xylanase XYNB and the N-terminus mutant TB have serine and asparagine, both of which can form hydrogen bonds, at the corresponding sites of the disulfide bridge. However, a covalent disulfide bond is more stable than a hydrogen bond, and hydrogen bond formation is more readily disrupted by pH changes compared with disulfide bonds [13]. This may explain why XS and TS were more stable than XYNB and TB at acidic pH. In addition, introduction of covalent disulfide bonds increases the rigidity of the folded molecule [4], which may increase the thermostability of the mutants. Indeed, the mutants containing disulfide bridges will be less flexible. The flexibility correlated directly with the enzymatic activity, which explains why the specific activities of TS and XS were lower than that of TB and XYNB (Table 1), and the K_m and the V_{max} values of the two mutants generally corresponded with the flexibility and activity of xylanases.

In conclusion, the synergism of N-terminus replacement and introduction of a disulfide bridge was responsible for increasing the thermostability of TS by about 12.4-fold when compared with XYNB at 70°C, pH 6.0, for 20 min, and the half-life of XYNB increased from about 3 to 150 min. The resultant mutant TS is potentially useful for industrial applications. Moreover, the mutants generated in this study will be instrumental for further research on the relationship between the structure and function of xylanases.

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