APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2010, p. 356–360 Vol. 76, No. 1
0099-2240/10/$12.00 doi:10.1128/AEM.00357-09

Effect of Glycosylation and Additional Domains on the Thermostability of a Family 10 Xylanase Produced by *Thermopolyspora flexuosa*▽†

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Received 13 February 2009/Accepted 19 October 2009

The effects of different structural features on the thermostability of *Thermopolyspora flexuosa* xylanase XYN10A were investigated. A C-terminal carbohydrate binding module had only a slight effect, whereas a polyhistidine tag increased the thermostability of XYN10A xylanase. In contrast, glycosylation at Asn26, located in an exposed loop, decreased the thermostability of the xylanase. The presence of a substrate increased stability mainly at low pH.

The thermophilic actinomycete *Thermopolyspora flexuosa*, previously named *Nonomuraea flexuosa* and before that *Actinomadura flexuosa* or *Microtetraspora flexuosa* (15), produces family 11 and family 10 xylanases, which show high thermostability (16, 17, 22). *T. flexuosa* xylanase XYN10A has a C-terminal family 13 carbohydrate binding module (CBM) (22). Many xylanases have an additional CBM, which can be a cellulose binding domain (CBD) or a xylan binding domain (XBD) (1, 5, 7, 22, 25, 28). XBD typically increases activity against insoluble xylan (1, 5, 24), although some XBDs also bind soluble xylans (21, 25).

We studied the thermostability of *T. flexuosa* xylanase XYN10A and how CBM and other additional groups affect its thermostability. In addition to confirming the previously described importance of terminal regions, our study identified a loop that is important for the thermostability of *T. flexuosa* XYN10A. In general, identification of sites important for protein stability is necessary for targeted mutagenesis attempts to increase thermostability.

The *T. flexuosa xyn10A* gene (GenBank accession no. AJ508953) (22), which encodes the full-length XYN10A xylanase (1-AAST . . . SYNA-448) containing the catalytic domain and CBM, and a truncated gene, which encodes the catalytic domain only (1-AAST . . . DALN-301) were expressed in *Trichoderma reesei* as 3′ fusions to a sequence that encodes the Cel6A CBD (A+B) carrier polypeptide and a Kex2 cleavage site (RDKR) (27). In this article, the catalytic domain and the full-length enzyme are referred to as XYN10A and XYN10A-CBM, respectively. The catalytic domain was also produced in *Escherichia coli*. For production in *E. coli*, the sequence encoding the catalytic domain was cloned into a pKKtac vector (33) with and without an additional 3′ sequence encoding a 6×His tag at the protein C terminus (. . . DALNH HHHHH).

The proteins were purified by hydrophobic interaction chromatography using a Phenyl Sepharose column and by ion-exchange chromatography using a DEAE Sepharose FF column (Amersham Pharmacia Biotech). The 6×His-tagged XYN10A xylanase produced in *E. coli* was purified by affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen).

Mass spectrometric (MS) analyses were performed on a high-resolution 4.7-T hybrid quadrupole-Fourier transform ion cyclotron resonance (FT-ICR) instrument (APEX-Qe; Bruker Daltonics), which employs electrospray ionization (ESI) (see supplemental material for details).

Xylanase activity was measured with a 3,5-dinitrosalicylic acid assay by using 1% solubilized birchwood xylan as a substrate (33). The optimum temperature, residual activity, and half-life assays were performed as described earlier (36). SWISS-MODEL (4) was used to automatically model *T. flexuosa* XYN10A and XYN10A-CBM (PDB codes for the modeling templates are 1v6w and 1e0w, respectively [12, 14]).

The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that the masses of XYN10A xylanase and XYN10A-CBM produced in *Trichoderma reesei* were ~37 kDa and ~50 kDa, respectively (Fig. 1A). MS analysis of the 6×His-tagged XYN10A produced in *E. coli* (SDS-PAGE not shown) indicated the presence of a single protein form (Fig. 1B), with a measured mass of 34,943.25 Da. This is consistent with the theoretical mass of 6×His-tagged XYN10A (34,942.93 Da). In contrast, XYN10A produced in *T. reesei* was heterogeneously modified, and six protein forms (numbered 1 to 6) were detected (Fig. 1B). The mass of form 1 (34,120.76 Da) is in excellent agreement with the calculated mass of XYN10A (34120.73 Da). The masses of forms 2 and 3, with mass increments of ~203 and ~162 Da, respectively, suggested protein glycosylation (+203 Da = GlcNAc; +162 Da = Man). There are two potential sites for *N*-glycosylation

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 23 October 2009.

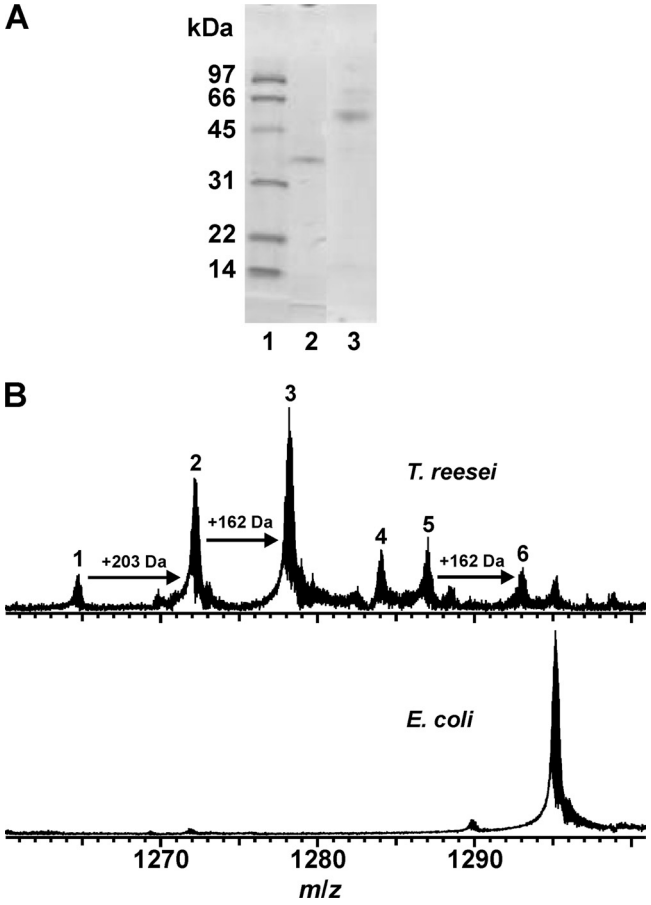

FIG. 1. (A) SDS-PAGE of purified XYN10A and XYN10A-CBM produced in *Trichoderma reesei*. Lane 1, molecular weight markers; lane 2, catalytic domain (XYN10A); lane 3, full-length enzyme (XYN10A-CBM). (B) ESI FT-ICR mass spectra of XYN10A with a 6×His tag produced in *E. coli* (bottom) and XYN10A produced in *T. reesei* (top). Only the expanded view at *m/z* 1260 to 1300, with the signals representing the most abundant protein ion charge state $z = 27+$, is presented. For the measured and calculated masses of the protein forms identified, see the supplemental material.

in XYN10A, Asn26 and Asn95. These six protein forms were resolved only by the high-resolution FT-ICR MS technique, not by SDS-PAGE (eluted as a single band [Fig. 1A]).

In order to locate the glycosylation site or sites, XYN10A proteins produced in *E. coli* and *T. reesei* were subjected to on-line pepsin digestion (see supplemental material for details). The sequence coverage for XYN10A xylanase produced in *E. coli* was 62%. For XYN10A produced in *T. reesei*, a lower sequence coverage was obtained, but three glycopeptides (residues 20 to 44, 20 to 46, and 20 to 59), carrying one GlcNAc residue, were detected (glycopeptides A to C in Fig. S1B in the supplemental material). A triply charged glycopeptide A was further analyzed by collision-induced dissociation (CID) measurement (see inset in Fig. S1B in the supplemental material). A ladder of *b*-type fragment ions further identified this peptide and verified Asn26 as the *N*-glycosylation site in XYN10A, carrying GlcNAc(Man) as a glycan core structure.

The additional sequences attached to the catalytic domain affected the thermostability of XYN10A xylanase. The deletion

TABLE 1. Peaks of the optimum temperatures (30-min assay)[a]

Production host	Enzyme	Optimum temp (°C) at:		
		pH 5.5	pH 7	pH 8.5
T. reesei	XYN10A	70	70	69
	XYN10A-CBM	70	72	72
E. coli	XYN10A	78	75	76
	XYN10A-6×His	78	78	78

[a] One percent solubilized birchwood xylan was used as the substrate in the assay.

of the native C-terminal CBM domain (XYN10A produced in *T. reesei*) slightly decreased (~2°C) the apparent temperature optimum in the region of 70 to 75°C (Table 1 and Fig. 2A). However, at 80°C, the deletion of the CBM domain increased the activity (Fig. 2A). Furthermore, the half-life in the pres-

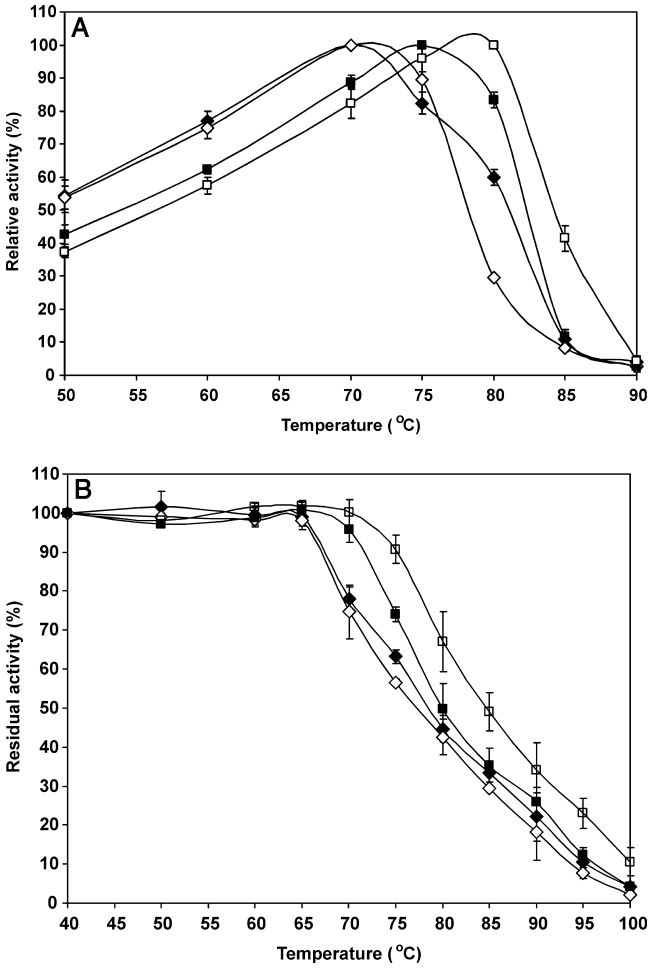

FIG. 2. Enzyme activity and stability profiles. (A) Enzyme activity as a function of temperature. The enzymes were incubated for 30 min at each temperature at pH 7. (B) Enzyme inactivation as a function of temperature. The enzyme samples were incubated without the substrate for 30 min at each temperature (pH 7), and the residual activity was measured at 70°C. Values are means ± standard deviations (error bars) for three experiments. Symbols: ◆, XYN10A xylanase produced in *T. reesei*; ◇, XYN10A-CBM produced in *T. reesei*; ■, XYN10A produced in *E. coli*; □, XYN10A-6×His produced in *E. coli*.

TABLE 2. pH-dependent half-life times of a catalytic domain (XYN10A) and a full-length enzyme (XYN10A-CBM) produced in *T. reesei*

Enzyme	Half-life (min) of enzyme under various conditions							
	With substrate[a]				Without substrate			
	pH 4 and 65°C	pH 5.5 and 80°C	pH 7 and 80°C	pH 8.5 and 80°C	pH 4 and 65°C	pH 5.5 and 80°C	pH 7 and 80°C	pH 8.5 and 80°C
XYN10A	18	37	37	33	3.1	19	23	23
XYN10A-CBM	15	17	17	14	1.3	33	22	26

[a] One percent solubilized birchwood xylan was used as the substrate in the assay.

ence of the substrate at 80°C was lower when the CBM was present (Table 2).

Surprisingly, the apparent temperature optimum of XYN10A xylanase produced in *E. coli* was 4 to 8°C higher than that for XYN10A produced in *T. reesei* (Fig. 2A and Table 1). In addition, the C-terminal 6×His tag further increased the apparent temperature optimum of XYN10A by ~3°C at pH 7 and 8.5 (Fig. 2A). The higher stability of XYN10A produced in *E. coli* was also seen in the residual activity profiles (Fig. 2B). However, the 6×His tag did not elevate the temperature optimum at pH 5.5 (Table 1) and pH 4.0 (not shown).

We also measured the enzyme half-lives with and without substrate (1% solubilized birchwood xylan) at different pH values. Increases of about 5- to 10-fold in the half-lives of both XYN10A xylanase and XYN10A-CBM (produced in *T. reesei*) were measured at pH 4 in the presence of a substrate (Table 2). The substrate also slightly protected XYN10A in the pH range from pH 5.5 to 8.5. However, no protection by the substrate was detected for XYN10A-CBM at pH 5.5 to 8.5.

By comparing the structures of thermophilic and mesophilic family 10 xylanases, it was suggested that efficient packing of the hydrophobic core, favorable charge interactions with the helix dipole moment, and the presence of prolines at the N termini of alpha-helices are the most probable stabilizing factors (23). Cavity filling and stabilization of loops and N- and C-terminal regions are also important factors (2, 35). By studying chimeric xylanase created by the shuffling of *Thermotoga maritima* xylanases A and B, it was observed that the N-terminal and C-terminal regions of the xylanase structure formed from the TIM barrel are important for high thermostability (20). Our results also showed that the C-terminal region is important for the thermostability of family 10 xylanases.

An increase in the thermostability of other proteins by a polyhistidine tag has already been demonstrated (8, 9, 10, 19). In *T. flexuosa* XYN10A xylanase, the 6×His tag had an effect on thermostability only at a neutral or alkaline pH. Since histidine is generally neutral in charge above pH 6.5 (average pK_a about 6.5) and positively charged at acidic pH, this suggests that noncharged interactions are critical for the stabilization effect.

The binding of the C-terminal 6×His tag to the surface of XYN10A xylanase probably prevents unfolding from the C terminus. The disulfide bridge between the N and C termini (located close to each other) has previously been demonstrated to increase the melting temperature (T_m) of a family 10 xylanase by 4°C (2, 35). The thermostability increase achieved by the 6×His tag and CBM in *T. flexuosa* XYN10A was at the same level (in the range of 3°C in the activity assays). Other stabilization mechanisms are also possible, but it seems probable that the role of protein termini is dominant in stabilization by the 6×His tag. The stability of alpha-helices near the C terminus could also be increased by interaction with the 6×His tag (Fig. 3).

Structural modeling was used to examine the regions potentially binding the 6×His tag. In the crystal and nuclear magnetic resonance (NMR) structures 1ddf, 1jt3, and 1zu2, the length of the 6×His tag varies between 12 and 20 Å, since the conformation of the freely protruding 6×His tag may vary significantly. Thus, the 6×His tag forms a rather large binding surface with much variation in the conformation. Since the stabilizing effect of the 6×His tag is pH dependent, it could be that the nearby arginines, having positive charges, have a role in breaking the interactions of the polyhistidine when it becomes positively charged at low pH (Fig. 3). Three nearby arginines (Arg14, Arg219, and Arg252) and a histidine (His12) in the 12-Å distance range from the first histidine in the 6×His tag might cause charge repulsion, and Arg36 and Lys289 at a distance of 17 to 20 Å in the opposite direction might also cause similar repulsion (Fig. 3).

The glycosylation site (Asn26) is located in a well-exposed loop (amino acids 21 to 28) between a beta-strand (amino acids 15 to 20) and alpha-helix (amino acids 29 to 37). Glycosylation can increase the thermostability (6, 18, 29). It can also destabilize, and, according to molecular dynamics simulations, increased mobility correlates with the destabilization caused by glycosylation (31). Glycosylation in a well-exposed loop in XYN10A xylanase could increase local mobility or destabilize the enzyme by affecting the local conformation.

The presence of a substrate increased the stability of both the core and full-length XYN10A xylanase under stronger acidic conditions of pH 4 (Table 2). At pH 5.5 to 8.5, the relative effect was smaller for the XYN10A core and missing in XYN10A-CBM. Protection by a substrate, especially at acidic pH, was observed by Xiong et al. (36) for a family 11 xylanase produced by *Thermomyces lanuginosus*. A possible explanation for this is that the substrate changes the structure of the enzyme or is involved in hydrogen bonding in the active site in a pH-dependent manner. At pH 4, in which the carboxylic acids start to become on average protonated and the ion pair networks are therefore disturbed, the thermostability of the enzyme is lower than at higher pH. Thus, the substrate could partially neutralize the lower thermostability at low pH by providing new stabilizing interactions. These results suggest that the active site canyon is also important for the stability of xylanases.

The effect of the CBM on the thermostability of XYN10A

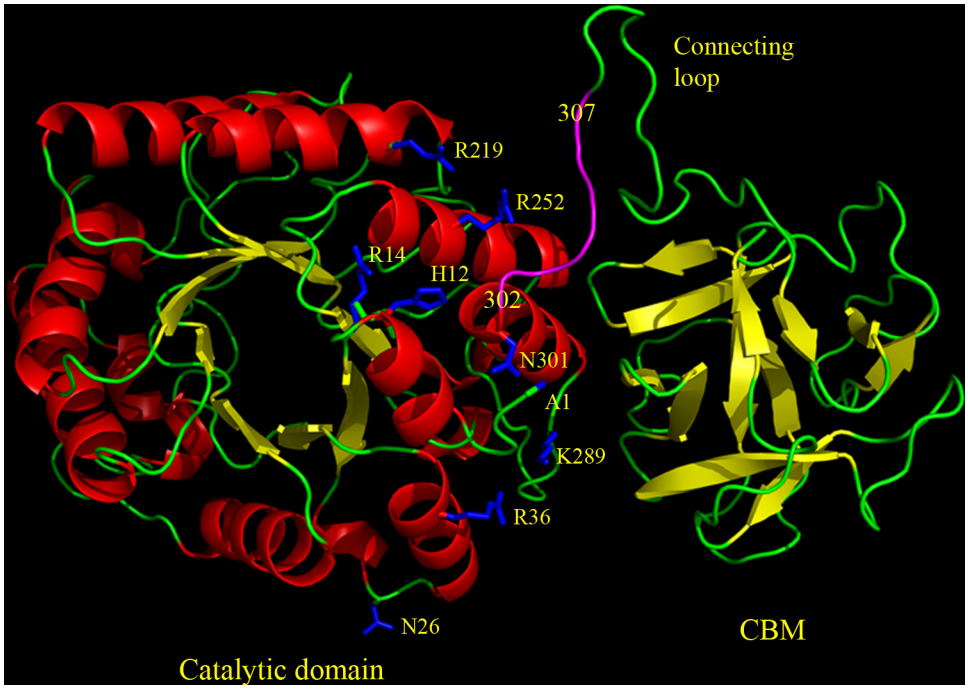

FIG. 3. Modeled structure of full-length XYN10A xylanase. The model was created by SWISS-MODEL using 1v6w as a template, and the figure was made using PyMOL (11). The residue Asn301 is the C terminus of the expressed catalytic core. The residue Ala1 (A1) shows the position of the N terminus. The glycosylation site Asn26 and the positively charged residues (His12, Arg14, Arg36, Arg219, Arg252, and Lys289) in the range of the 6×His tag are shown as one-letter codes. The sequence positions corresponding to the 6×His tag (positions 302 to 307 in full-length XYN10A) are shown in magenta, although the conformation of the 6×His tag is not known. The active site is located on the other side of the barrel.

xylanase was twofold; under some conditions, it increased the thermostability, and under other conditions, it decreased the thermostability. Thus, there is no strong thermostabilizing effect by the CBM on *T. flexuosa* XYN10A. It was observed earlier that the additional domains may function as thermostabilizing domains, because their deletion often decreased the stability of xylanases (3, 30, 32). However, an increase in thermostability has also been observed when a CBM has been deleted (3, 22, 23a, 26). Thus, the effect of a CBM on thermostability varies, and the reason could be that the primary function of a CBM is to bind polysaccharide fibers and not thermostabilization. In general, the high thermostability of xylanases is not dependent on CBMs, and in fact, they might have diverse effects. The same holds true for protein glycosylations.

In conclusion, we identified several regions in *T. flexuosa* XYN10A xylanase that affect the protein's thermostability. The effects of the additional groups were either stabilizing or destabilizing. This information can be used in the design of stabilizing mutations. Our study also showed that the production system can considerably affect the properties of the enzymes produced, e.g., due to glycosylation, and that when adding purification tags in recombinant proteins, their potential effects should be considered.

We thank Johanna Aura for technical assistance, Antti Nyyssölä for assistance in protein purification, and Jarno Kallio, Sanna Hiljanen-Berg, and Sirpa Okko for *T. reesei* strain fermentation.

Financial support from the Research Foundation of the Helsinki University of Technology and the Academy of Finland is gratefully acknowledged.

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