Extremophiles (2004) 8:393–400
DOI 10.1007/s00792-004-0400-9

ORIGINAL PAPER

Hairong Xiong · Fred Fenel · Matti Leisola
Ossi Turunen

Engineering the thermostability of *Trichoderma reesei* endo-1,4-β-xylanase II by combination of disulphide bridges

Received: 6 February 2004 / Accepted: 10 May 2004 / Published online: 20 July 2004

Abstract Disulphide bridges were introduced in different combinations into the N-terminal region and the single α-helix of mesophilic *Trichoderma reesei* xylanase II (TRX II). We used earlier disulphide-bridge data and designed new disulphide bridges for the combination mutants. The most stable mutant contained two disulphide bridges (between positions 2 and 28 and between positions 110 and 154, respectively) and the mutations N11D, N38E, and Q162H. With a half-life of ~56 h at 65°C, the thermostability of this sevenfold mutant was ~5,000 times higher than that of TRX II, and the half-life was 25 min even at 75°C. The thermostability of this mutant was ~30 times higher than that of the corresponding mutant missing the bridge between positions 2 and 28. The extensive stabilization at two protein regions did not alter the kinetic properties of the sevenfold mutant from that of the wild-type TRX II. The combination of disulphide bridges enhanced significantly the pH-dependent stability in a wide pH range.

Keywords Endo-1,4-β-xylanase · Disulphide bridge · Thermostability · pH-dependent stability · Protein engineering

Introduction

Endoxylanases (EC 3.2.1.8.) hydrolyze by acid–base catalysis 1,4-β-D-xylosidic linkages in xylans. Xylanases are used for biomass modification in pulp bleaching, baking, and animal feeding (Viikari et al. 1994; Prade 1996). Xylanases encounter extreme conditions (high temperature, alkaline pH) in some of the applications. For this reason, natively thermophilic xylanases are potentially very useful in industrial applications. An alternative is to stabilize by protein engineering a mesophilic enzyme that has already been adopted for industrial use.

In family 11 xylanases (previously family G), two large β-pleated sheets and one α-helix form a structure that resembles a partially closed right hand (Törrönen and Rouvinen 1997). Structures of several mesophilic and thermophilic xylanases have been determined (Hakulinen et al. 2003). The thermostability of family 11 xylanases has been studied extensively, and improved stability has been achieved by a large number of mutations at different positions in the protein structure (Arase et al. 1993; Wakarchuk et al. 1994; Georis et al. 2000; Shibuya et al. 2000; Turunen et al. 2001, 2002; Sung 2003; Fenel et al. 2004). Moreover, the activity under alkaline conditions has been improved by site-directed mutagenesis and directed evolution (Chen et al. 2001; Turunen et al. 2002).

Disulphide bridges exist naturally in xylanases. Thermophilic *Paecilomyces varioti* and *Thermomyces lanuginosus* xylanases (PDB codes 1PVX and 1YNA, respectively) each have a disulphide bridge that cross-links the β-strand B9 to the α-helix (Grüber et al. 1998; Kumar et al. 2000). The site of this bridge corresponds the disulphide bridge introduced by site-directed mutagenesis between positions 110 and 154 in *Trichoderma reesei* XYNII and between positions 100 and 148 in *Bacillus circulans* xylanase, and the stabilizing effect of these bridges was confirmed experimentally (Wakarchuk et al. 1994; Turunen et al. 2001). However, the role of the disulphide bridge at the cord region is not known; the bridge is close to one end of the catalytic canyon. The mesophilic *Aspergillus kawachii* and *Aspergillus niger* xylanases (PDB codes 1BK1 and 1UKR, respectively) have this disulphide bridge that is located between the cord and the β-strand B8 (Krengel and Dijkstra 1996; Fushinobu et al. 1998). The active site

Communicated by G. Antranikian

H. Xiong · M. Leisola · O. Turunen (✉)
Laboratory of Bioprocess Engineering,
Helsinki University of Technology, P.O. Box 6100,
02015-HUT Helsinki, Finland
E-mail: Ossi.Turunen@hut.fi
Tel.: +358-9-4512551
Fax: +358-9-462373

F. Fenel
Carbozyme, Ltd., Keilaranta 16, 02150 Espoo,
Finland

cleft of the highly thermostable *Dictyoglomus thermophilum* xylanase (PDB code 1F5 J) contains two cysteines that might form a bridge, but the bridge was not seen in the crystal structure (McCarthy et al. 2000). Structurally, family 11 xylanases resemble family 12 endoglucanases. Most family 12 endoglucanases contain a disulphide bridge in the protein N-terminus. In *T. reesei* EG3, the bridge is located between positions 4 and 32 (Sandgren et al. 2001). No member of family 11 xylanases is known to have a corresponding N-terminal disulphide bridge. Experimentally, *B. circulans* xylanase has been stabilized by a disulphide bridge between the N-terminus and extended C-terminus (Wakarchuk et al. 1994) and *T. reesei* xylanase II (TRX II) by a disulphide bridge between positions 2 and 28 (Fenel et al. 2004). In *B. circulans* xylanase, the combination of the disulphide bridge between the N- and C-termini and the bridge between the α-helix and adjacent β-strand increased the thermostability cumulatively (Wakarchuk et al. 1994).

Genetically engineered additional disulphide bridges can increase the thermostability by 5–30°C (Pace 1990). Although oxidation of cysteines is a danger at temperatures above 80°C (Daniel et al. 1996), a genetically engineered disulphide bridge has been shown to stabilize a protein even at 100°C (Van den Burg et al. 1998). However, the stabilization by a disulphide bridge is not always successful. The location of the bridge is important. TRX II is not thermostable; it is stable at 45°C, and temperatures above 50°C cause conformational changes in the protein, and the enzyme activity is lost quickly (Tenkanen et al. 1992; Jänis et al. 2001; Turunen et al. 2001). In this study, we used TRX II to study how different combinations of disulphide bridges at the N-terminus and those between the single α-helix and β-strand B9 affect the thermostability and pH-dependent properties of the enzyme.

Materials and methods

Generation of TRX II mutants

In planning the mutations, Swiss-PdbViewer (http://www.expasy.ch/spdbv/; Guex and Peitsch 1997) was used as a tool to examine the TRX II structure (1xyp). The mutations were generated by polymerase chain reaction (PCR), in which the mutations were introduced into the oligonucleotide primers as described earlier (Turunen et al. 2001). The mutant clones were grown on agar plates containing xylan (birchwood xylan, Sigma, Steinheim, Germany) coupled to Remazol Brilliant Blue, in which the xylanase activity is indicated by white halos around the positive colonies (Biely et al. 1985).

Expression of TRX II

We used the *Trichoderma reesei* TRX II clone pALK143 as the expression construct as described earlier (Turunen et al. 2001). The xylanase was expressed from pALK143 under the control of *Bacillus amyloliquefaciens* α-amylase promoter, and the protein was secreted into the medium by *B. amyloliquefaciens* α-amylase signal sequence. Xylanases were purified for the determination of kinetic parameters as described earlier (Turunen et al. 2001).

Enzyme assay

Xylanase activity was determined by measuring with DNS assay the amount of reducing sugars liberated from 1% birchwood xylan (Bailey et al. 1992). The activity determination under standard conditions was carried out at 50°C (pH 5–6), with a reaction time of 10 min. Citrate–phosphate (50 mM) buffer was used in the xylanase assays at pH 4–7 and 50 mM Tris-HCl at pH 8. Growth medium or citrate–phosphate buffer was used to dilute the enzyme for activity and stability measurements. The half-lives (t_{half}) of the enzymes were determined at the pH region promoting highest enzyme activity (pH_{opt}), in which the enzymes showed a high stability, and thus: for TRX II at pH 5–6; for DS1 at pH 5; for DS3, DS4, and DB2 at pH 6; and for DS2, DS5, DB1, and DB3 at pH 8. The thermostability assays require long incubations in the test tubes, and diluted pure xylanases may disappear quite quickly from the solution. Thus, most properties of the mutant xylanases were tested using nonpurified enzymes (*Escherichia coli* culture broth). The results are thus relative, but comparable. It has also to be remembered that xylanase activities are always somewhat relative since the used substrate, xylan, is not a uniform molecule and its nature may vary from batch to batch.

Results

The mutants

Disulphide bridges were introduced between the α-helix and the adjacent β-strand B9 and into the N-terminal region to bind it more tightly to the protein core (see Fig. 1). Several alternative disulphide bridges were designed to stabilize these two major regions that are thought to affect the thermostability of family 11 xylanases. The disulphide bridges C2–C28 (Fenel et al. 2004), C110–C154 (Wakarchuk et al. 1994; Turunen et al. 2001;) and C108–C158 (corresponding bridge in *Bacillus circulans* xylanase is described by Wakarchuk et al. 1994) have been described before, and C7–C16 and C105–C162 are new bridges. We investigated the optimal way to combine different bridges using a few examples. The ability of each cysteine pair to form a disulphide bridge was predicted by Swiss-PdbViewer. The program showed that the side chains of each cysteine pair are in correct position to form a bridge. The

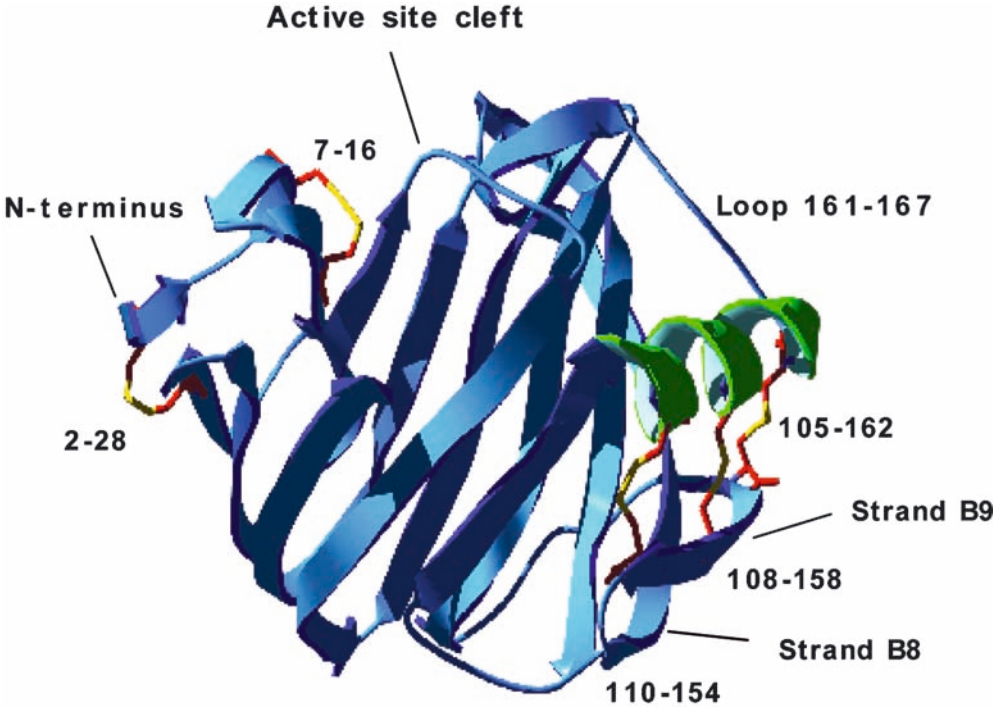

Fig. 1 Structure of *Trichoderma reesei* xylanase II (TRX II) (1xyp). *Numbers* indicate the positions of disulphide bridges (the corresponding residues are shown in *red*). The α-helix is shown in *green* and the β-strands are shown in *light blue*. The picture was created by Swiss-PdbViewer

significant increase in the thermostability was regarded as an indication that the disulphide bridges were formed in the protein. The presence of the disulphide bridges in the mutants DS2, DS5, and DB1 was verified for purified enzymes by mass spectrometry (Jänis et al. 2004). The list of mutants is shown in Table 1.

In the α-helix (amino acids 153–162), a disulphide bridge was introduced into the N-terminal end (position 154), middle region (position 158), or C-terminal end (position 162) [the basic properties of two first bridges were reported in other studies (Wakarchuk et al. 1994; Turunen et al. 2001)]. The mutant DS2 is the same as the mutant S, and the mutant DS5 the same as the mutant SHDE in our earlier study (Turunen et al. 2001). The protein N-terminus was bound more tightly to the protein core (double-layered β-sheet) by two alternative disulphide bridges (between positions 2 and 28 and 7 and 16, respectively). The basic properties of the C2–C28 bridge mutant Y5 (contained also the mutations K58R and +191D) were reported earlier (Fenel et al. 2004). The unintentional mutation Y27F was found in the mutants containing the C2–C28 bridge (except the mutant Y5). Because the thermostability of Y5 (Fenel et al. 2004) and DS1 were on the same level, Y27F is not expected to have any major effect on the stability in the mutants that contain the C2–C28 bridge. Two disulphide bridges at the N-terminus and two disulphide bridges at the α-helix, respectively, were combined in different combinations to see whether they all have a cumulative effect on xylanase stability. The temperature and pH-dependent properties of the mutants are summarized in Table 2.

Disulphide bridges at the α-helix

We compared the properties of three disulphide bridges located at different positions of the α-helix. The

Table 1 Mutants of *Trichoderma reesei* xylanase II (TRX II). *Underlined text* indicates the disulphide bridges. *DS* Disulphide bridge, *DB* double bridge, *Y27F* unintentional mutation

Code	Mutations	Reference
DS1	T2C, T28C, (Y27F)	
DS2	S110C, N154C	Turunen et al. (2001)[a] Wakarchuk et al. (1994)[b]
DS3	V108C, A158C	Wakarchuk et al. (1994)[b]
DS4	L105C, Q162C	
DS5	S110C, N154C, N11D, N38E, Q162H	Turunen et al. (2001)[a]
DB1	T2C, T28C, (Y27F), S110C, N154C, N11D, N38E, Q162H	
DB2	T2C, T28C, (Y27F), K58R, L105C, Q162C, +191D	
DB3	T7C, S16C, S110C, N154C, N11D, N38E, Q162H	
Y5	T2C, T28C, K58R, +191D	Fenel et al. (2004)

[a]DS2 was previously S, and DS5 was previously SHDE
[b]Corresponding mutations in *Bacillus circulans* xylanase

Table 2 Summary of the properties of TRX II mutants. T_{opt} is the apparent temperature optimum in the region that showed ≥90% of the maximal activity at pH 5 (see Fig. 3). T_{50} is the temperature at which 50% of the activity is left after 10-min incubation, done here at pH 6 (see Fig. 2). t_{half} is the half-life determined without the presence of substrate at 65°C: pH 5 for DS1; pH 6 for wild type, DS3, DS4, and DB2; and pH 8 for DS2, DB1, and DB3. pH_{opt} is the pH region that showed ≥90% of the maximal activity at 50°C (see Fig. 5). *High pH stability* indicates pH region that showed ≥90% of maximal stability (see Fig. 4)

	T_{opt} (°C)	T_{50} (°C)	t_{half}	pH_{opt}	High pH stability
Wild type	56–62	53	~0.5 min	4.8–6.4	5.0–6.8
DS1	65–75	67	30 min	4.8–6.6	4.2–5.3
DS2	52–59	63	9 min	4.8–6.4	6.9–9.1
DS3	53–59	57.5	< 1 min	4.9–6.4	5.6–6.4
DS4	50–57	57.5	< 1 min	4.8–6.1	4.8–6.2
DB1	63–74	76	≥56 h	4.9–6.4	5.0–9.2
DB2	61–70	66	26 min	5.0–6.3	3.8–6.0
DB3	50–60	69	≥6 h	4.8–5.5	4.8–9.0

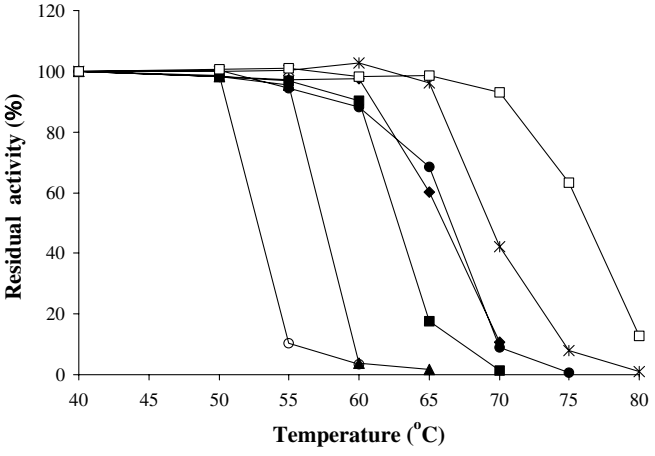

Fig. 2 Inactivation of the enzyme as a function of temperature. The enzyme samples were heated without the presence of substrate for 10 min at each temperature (pH 6), and the residual activity was measured at 50°C. *Open circle* TRX II, *filled circle* DS1, *filled square* DS2, *filled triangle* DS4, *open square* DB1, *filled diamond* DB2, *asterisk* DB3. The profile of DS3 was the same as that of DS4 (not shown)

disulphide bridge DS2 gave a better stability against inactivation at high temperatures than the disulphide bridges DS3 and DS4 (Fig. 2; Table 2). The same was observed in *B. circulans* xylanase for the disulphide bridges corresponding to DS2 and DS3 (Wakarchuk et al. 1994). Although the thermostability of all three disulphide bridge mutants was higher, the apparent temperature optimum (T_{opt}) was slightly lower than that of TRX II (Fig. 3; Table 2). Thus, these disulphide bridges also had some adverse effects on the protein. The position of the disulphide bridge affected the enzyme's properties (Figs. 4a, 5). DS4 had a slightly lower pH_{opt} than DS2, DS3, and TRX II (Fig. 5). pH-dependent stability of DS2 was highest at ~pH8; DS4 was most stable at ~pH5.5 and DS3 at ~pH6 (Fig. 4a; Table 2).

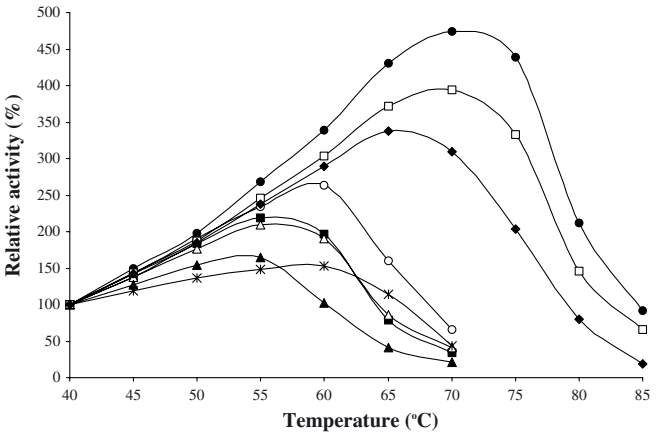

Fig. 3 The apparent temperature optimum profiles. The enzyme activity was measured by 10-min assay at each temperature (pH 5). The activity at 40°C is shown as 100%. *Open circle* TRX II, *filled circle* DS1, *filled square* DS2, *open triangle* DS3, *filled triangle* DS4, *open square* DB1, *filled diamond* DB2, *asterisk* DB3

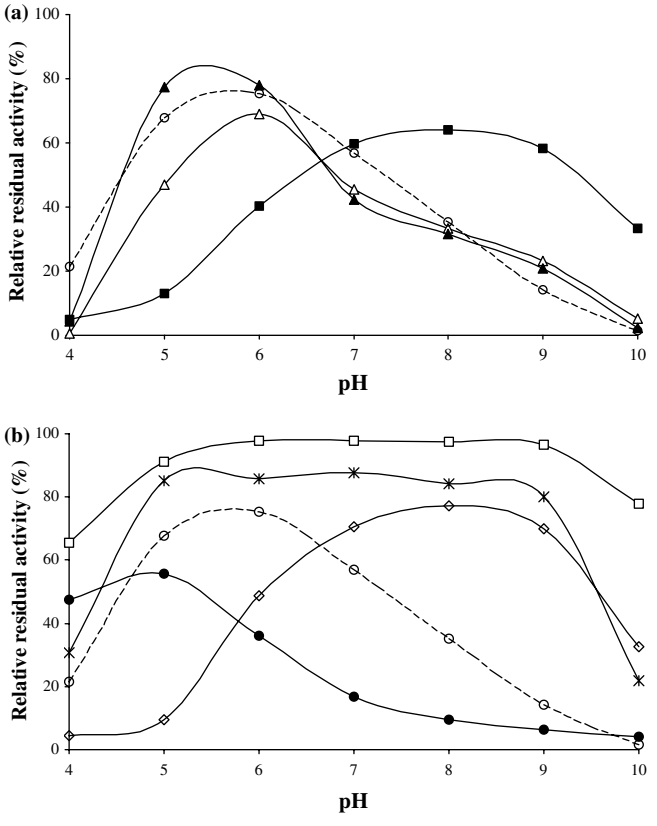

Fig. 4a, b pH-dependent stability profiles. **a** The enzyme samples were incubated for 30 min at 52.5°C (TRX II), 55°C (DS3 and DS4) or 60°C (DS2) before the measurement of residual activity at 50°C. *Open circle* TRX II; *filled square* DS2, *open triangle* DS3, *filled triangle* DS4. **b** The enzyme samples were incubated for 30 min at 52.5°C (TRX II) or 65°C (all mutants in **b**). *Open circle* TRX II, *filled circle* DS1, *open diamond* DS5, *open square* DB1, *asterisk* DB3

Thus, the pH-dependent stability profile of each of these disulphide bridge mutants was determined by the position of the bridge at the α-helix.

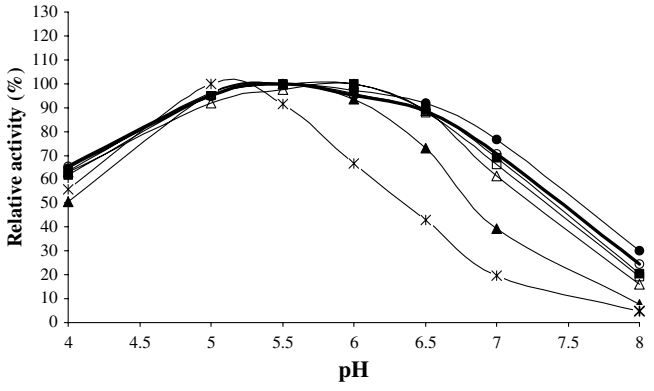

Fig. 5 pH-dependent activity profiles at 50°C. *Open circle* TRX II (*thick line*), *filled circle* DS1, *filled square* DS2 *open triangle* DS3, *filled triangle* DS4, *open square* DB1, *asterisk* DB3

Combination of disulphide bridges at the α-helix and N-terminal region

The C2–C28 bridge mutant DS1 had 13–14°C higher thermostability than TRX II (Figs. 2, 3; Table 2). The C2–C28 bridge elevated both the tolerance against inactivation at high temperatures and the T_{opt} (Figs. 2, 3; see also Fenel et al. 2004). The thermostability was increased further by introduction of the C2–C28 bridge into the DS2-based mutant DS5. In the DS5 mutant, the thermostability had been increased considerably by the mutations N11D, N38E, and Q162H from the stability level of the C110–C154 bridge (Turunen et al. 2001). The t_{half} of DS5 was 2 h at 65°C (Turunen et al. 2001). When the N-terminal disulphide bridge was introduced into this mutant, a hyperstable variant (DB1) of TRX II was created (see T_{50} and t_{half} in Table 2). The t_{half} of DB1 in the absence of the substrate was at least 56 h at 65°C (pH 8), 5–6 h at 70°C and 25 min at 75°C (Table 3). The t_{half} of TRX II was about 0.5 min at 65°C and 7 s or less at 70°C. Thus, the stability of DB1 was at least 5,000 times higher at 65°C than that of TRX II. The overall thermostability increased by 23°C (Fig. 2; Table 2) and the pH-dependent stability profile became very broad (Fig. 4b). When estimated from t_{half}, the thermostability of DB1 was ~30 and ~110 times higher than the thermostability of its parent mutants, DS5 and DS1, respectively.

The T_{opt} of DB1 remained at 63–74°C (pH 5), and thus, did not increase from the level of the DS1 mutant (65–75°C) (Fig. 3). In general, the T_{opt} of TRX II and its mutants was 2–3°C higher at pH 5 than at pH 6 (not shown). Although DB1 and DS1 had similar temperature optima when the incubation time was short in the enzyme assay (10 min, Fig. 3), in the extended incubations with the substrate (2 h or more), especially at pH 7, DB1 retained longer its activity than DS1 (Fig. 6). In the presence of the substrate, the t_{half} of DB1 was estimated from the time-dependent enzyme reaction curves to be in the range of 30–60 min at 75°C (pH 5) (not shown). The t_{half} without the substrate was 25 min at 75°C. Thus, the stabilizing effect of the substrate is quite small at these temperatures.

When the kinetic properties of DB1 were compared to TRX II (measured at 50°C, pH 5), it was found that both K_m and V_{max} values of DB1 were at the same level than those of TRX II (Table 3). That the mutations did not impair the enzyme is supported also by the finding that the Arrhenius activation energy (Doran 2000) of TRX II, DB1, as well as DS1, was about the same (~50 kJ/mol).

We tested whether the C105–C162 bridge at the C-terminal end of the α-helix increases the thermostability when combined with the mutations in Y5 (T2C, T28C, K58R, +191D), creating the mutant DB2. The result was that DB2 had, in fact, a lower T_{opt} (~66°C) than the C2–C28 bridge mutant DS1 or Y5 (70–71°C) (Fig. 3). The stability of DB2 against inactivation at high temperatures without the presence of substrate was about the same than that of the single disulphide bridge (C2–C28) mutants (see DS1 and DB2 in Fig. 2). Thus, the combination of disulphide bridges C2–C28 and C105–C162 did not have a cumulative effect on the thermostability.

Introduction of the C7–C16 bridge into the mutant DS5 (Turunen et al. 2001) also created a highly stable mutant (DB3) with a t_{half} of at least 6 h at 65°C (pH 8). In comparison, the t_{half} of DS5 was 2 h at 65°C (pH 8). Thus, the thermostability of DB3 was higher at pH 8 than that of the parent mutant DS5 (see also Fig. 4b). However, DB3 had a very wide pH-stability profile, and the improvement of the stability, compared to DS5, occurred primarily at pH region 5–7 (Fig. 4b). At pH 5, the t_{half} of DB3 was about 8 h, whereas the t_{half} of the parent mutant DS5 was only 8.5 min. The thermostability of DB3 was not as high as that of DB1 (Table 2); moreover, the T_{opt} increased only slightly from that of TRX II (Fig. 3). The pH-dependent activity profile showed that the activity of DB3 was remarkably decreased at pH region 6–8 (Fig. 5). The temperature-dependent activity profile of DB3 was quite different from that of TRX II and other mutants (Fig. 3). The Arrhenius activation energy of this mutant was remarkably lower (~25 kJ/mol) than that of TRX II (~50 kJ/mol). The K_m of the nonpurified DB3 was approximately three times higher than that of TRX II (not shown). Thus, the C7–C16 bridge increased the thermostability, but also had some negative effects on

Table 3 Comparison of TRX II and mutant DB1. Kinetic parameters were determined at 50°C (pH 5). *ND* Not determined

	TRX II	DB1
T_{50} (pH 6)	53°C	76°C
T_{opt} (pH 5)	56–62°C	63–74°C
t_{half} at 65°C	~0.5 min	~56 h
t_{half} at 70°C	≤ 7 s	5.75 h
t_{half} at 75°C	ND	25 min
K_m (mg/ml)	0.94	0.92
V_{max} (nkat/mg)	32,000	32,400

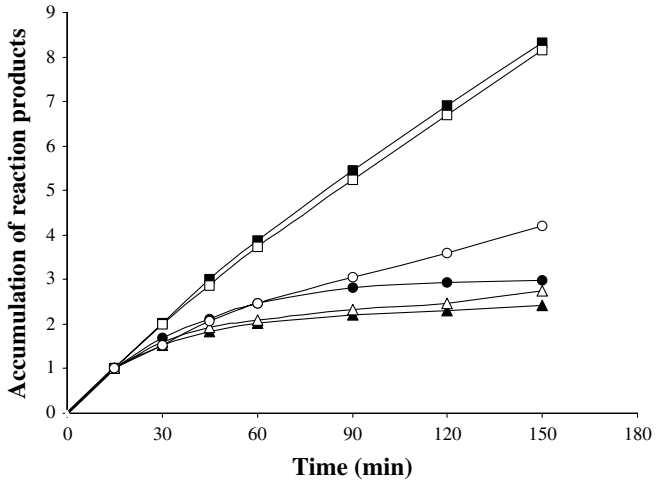

Fig. 6 Productivity assay of DS1 and DB1 mutants. The accumulation of the reaction product was followed as a function of time. *Filled square* DS1 at 65°C, pH 5; *open square* DB1 at 65°C, pH 5; *filled circle* DS1 at 65°C, pH 7; *open circle* DB1 at 65°C, pH 7; *filled triangle* DS1 at 75°C, pH 5; *open triangle* DB1 at 75°C, pH 5

the enzyme. In conclusion, the position of the disulphide bridge at the protein N-terminus is critical for the degree of stabilization and functional properties of xylanase.

Discussion

Disulphide bridges can be introduced into many positions in family 11 xylanases. The disulphide bridges are thought to stabilize the protein structure by decreasing the entropy of the unfolded state, though enthalpic and native-state effects should also be taken into consideration (Betz 1993; Vogl et al. 1995). The denaturation of the *Bacillus circulans* disulphide- bridge mutant, corresponding to the C110–C154 bridge in TRX II, has been studied by differential scanning calorimetry (Davoodi et al. 1998). The disulphide bridge in *B. circulans* xylanase increased the rate of refolding and also, but to a lesser extent, the unfolding rate. The net result was that the disulphide bridge forces the unfolded form to fold back to the native state (Davoodi et al. 1998). Molecular-dynamics simulations have been used to identify flexible regions in proteins, and subsequently, the protein stability has been increased considerably by introducing a disulphide bridge into such a region (Pikkemaat et al. 2002; Liu and Wang 2003). Engineered disulphide bridges in flexible regions may contribute more to a protein's thermostability than bridges in rigid regions (Tidor and Karplus 1993; Li et al. 1998; Liu and Wang 2003). In TRX II, the remarkably high thermostability gained by the disulphide bridge at the N-terminus or by the bridge between the α-helix and the β-strand B9 strongly suggests that these are the major regions in which the unfolding occurs early during protein denaturation.

An N-terminal disulphide bridge can be used to prevent the unfolding of xylanase structure by binding the N-terminus tightly to the protein core (β-sheet). In combination with the C110–C154 bridge (and other stabilizing substitutions), both the bridges C2–C28 and C7–C16 showed high stability over a broad pH range, though the stabilizing effect of the C2–C28 bridge was stronger. A major difference was that the mutant containing the C7–C16 bridge had a low T_{opt}, which is not much higher than that of TRX II. A reason for the difference between the stabilizing effects could be that the C7–C16 bridge allows the protein N-terminus to unfold partially (amino acids 1–6) at high temperatures. These results indicate that already a limited unfolding at the protein N-terminus is harmful for the enzyme activity. Another possible effect of the C7–C16 bridge located inside the active site cleft could be that it affects negatively the interaction of substrate with the enzyme. A higher K_m than that of TRX II supports this conclusion.

The combination of different disulphide bridges at α-helix–β-strand B9 and at the N-terminal region in our study showed that not all combinations have a cumulative effect on the thermostability. The reason for this is unclear. The differences between the stabilizing effects of different disulphide bridges at the α-helix is also unclear, though some possible reasons can be hypothesized. Residues 161–165 at the C-terminus of the α-helix (α-helix residues are 153–162) and the following loop (residues 161–167) show high B-factor values (1xyp). TRX II can be stabilized by binding the high B-factor region (positions 161–165) more tightly to the protein core, but this is not the best-known way to stabilize this protein area. The reason why the disulphide bridge in the beginning of the α-helix (at position 154) gives the highest stability may not be the stabilization of the high B-factor region. Instead, a possible reason for the higher stabilizing effect of the C110–C154 bridge could be that it prevents the unfolding of the nearby β-hairpin (strand B9, residues 103–110; and part of the strand B8, residues 113–118). The cross-link at the C-terminus of the α-helix (at position 162) is not as likely to have the same effect. An indication that the C110–C154 bridge is important for the stability of β-strands comes from a finding that the corresponding disulphide bridge in *Thermomyces lanuginosus* xylanase prevented the heat-induced unfolding of β-sheets (Tatu et al. 1990). Cleavage of the disulphide bridge caused a 25% loss of the β-strand structures in the studied conditions (Tatu et al. 1990).

Based on the inactivation patterns of the TRX II mutants, it is possible to make conclusions about reversible and irreversible inactivation of this enzyme. The disulphide bridges C2–C28 and C110–C154 appear to have different effects on the enzyme inactivation. Reversible inactivation is not seen in the experiments in which the enzyme is first heated at high temperatures, and then, the residual activity is measured at low temperature; this assay measures irreversible inactivation. Reversible inactivation can be detected by methods that

follow the changes at high temperatures themselves. Reversible inactivation at high temperatures modulates the T_{opt}. In TRX II, the C2–C28 bridge confers a higher activity at high temperatures, and the C110–C154 bridge does not have this property. The conclusion is that when the C110–C154 bridge stabilizes the α-helix and/or its nearby region, reversible inactivation at the protein N-terminus still occurs, which is reflected in the inability of the mutant enzyme to function at high temperatures. When the N-terminus is stabilized by the C2–C28 bridge, the reversible inactivation no longer has a dominating effect, and as a consequence, the T_{opt} becomes much higher. Thus, the comparison of the TRX II mutants reveals that the protein N-terminus, and not the α-helix region, is a major site for reversible inactivation. These results indicate that the unfolded N-terminus folds back to double-layered β-sheet structure when the denaturing conditions are removed.

In conclusion, the thermostability level achieved by the most optimal combination of disulphide bridges (containing also a few other mutations) was ~5,000-fold higher at 65°C than that of TRX II. The sevenfold mutant showed quite high stability even at 70–75°C, whereas TRX II is inactivated quickly above 50°C. Although this mutant is thermophilic by its nature, and the thermostability increased over 20°C from that of the wild type, its level of thermostability is still 15–20°C lower than the level of the most thermostably known family 11 xylanase of *Dictyoglomus thermophilum*, which is stable and active at 85°C (Morris et al. 1998). Consequently, the engineering of a mesophilic family 11 xylanase to be fully active at temperatures close to 100°C is still a great challenge.

Acknowledgements We thank Johanna Aura for technical assistance and Dr. Xiaoyan Wu for assistance in protein purification. Financial support from the Academy of Finland, the National Technology Agency of Finland, TEKES, and the Research Foundation of Helsinki University of Technology is gratefully acknowledged.

References

Arase A, Yomo T, Urabe I, Hata Y, Katsube Y, Okada H (1993) Stabilization of xylanase by random mutagenesis. FEBS Lett 316:123–127

Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270

Betz SF (1993) Disulfide bonds and the stability of globular proteins. Protein Sci 2:1551–1558

Biely P, Mislovicova D, Toman R (1985) Soluble chromogenic substrates for the assay of endo-1,4-β-xylanases and endo-1,4-β-glucanases. Anal Biochem 144:142–146

Chen Y-L, Tang T-Y, Cheng K-J (2001) Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase. Can J Microbiol 47:1088–1094

Daniel RM, Dines M, Petach HH (1996) The denaturation and degradation of stable enzymes at high temperatures. Biochem J 317:1–11

Davoodi J, Wakarchuk WW, Surewicz WK, Carey PR (1998) Scan-rate dependence in protein calorimetry: the reversible transitions of *Bacillus circulans* xylanase and a disulfide-bridge mutant. Protein Sci 7:1538–1544

Doran PM (2000) Bioprocess engineering principles. Academic Press, New York

Fenel F, Leisola M, Jänis J, Turunen O (2004) A de novo designed N-terminal disulfide bridge stabilizes the *Trichoderma reesei* endo-1,4-β-xylanase II. J Biotechnol 108:137–143

Fushinobu S, Ito K, Konno M, Wagaki T, Matsuzawa H (1998) Crystallographic and mutational analyses of an extremely acidophilic and acid-stable xylanase: biased distribution of acidic residues and importance of Asp37 for the catalysis at low pH. Protein Eng 11:1121–1128

Georis J, de Lemos Esteves F, Lamotte-Brasseur J, Bougnet V, Devreese B, Giannotta F, Granier B, Frére J-M (2000) An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. Protein Sci 9:466–475

Grüber K, Klintschar G, Hayn M, Schlacher A, Steiner W, Kratky C (1998) Thermophilic xylanase from *Thermomyces lanuginosus*: high-resolution X-ray structure and modeling studies. Biochemistry 37:13475–13485

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-Pdb-Viewer: an environment for comparative protein modelling. Electrophoresis 18:2714–2723

Hakulinen N, Turunen O, Jänis J, Leisola M, Rouvinen J (2003) Three-dimensional structures of thermophilic β-1,4-xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa*: comparison of twelve xylanases in relation to their thermal stability. Eur J Biochem 270:1399–1412

Jänis J, Rouvinen J, Leisola M, Turunen O, Vainiotalo P (2001) Thermostability of endo-1,4-β-xylanase II from *Trichoderma reesei* studied by electrospray ionization Fourier-transform ion cyclotron resonance MS, hydrogen/deuterium-exchange reactions and dynamic light scattering. Biochem J 356:453–460

Jänis J, Turunen O, Leisola M, Derrick P, Rouvinen J, Vainiotalo P (2004) Characterization of mutant xylanases using fourier transform ion cyclotron resonance mass spectrometry: stabilizing contributions of disulfide bridges and N-terminal extensions. Biochemistry (in press)

Krengel U, Dijkstra BW (1996) Three-dimensional structure of endo-1,4-β-xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. J Mol Biol 263:70–78

Kumar PR, Eswaramoorthy S, Vithayathil PJ, Viswamitra MA (2000) The tertiary structure at 1.59 Å resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *Paecilomyces varioti* Bainier. J Mol Biol 295:581–593

Li Y, Coutinho PM, Ford C (1998) Effect on thermostability and catalytic activity of introducing disulfide bonds into *Aspergillus awamori* glucoamylase. Protein Eng 11:661–667

Liu H-L, Wang W-C (2003) Protein engineering to improve the thermostability of glucoamylase from *Aspergillus awamori* based on molecular dynamics simulations. Protein Eng 16:19–25

McCarthy AA, Morris DD, Bergquist PL, Baker EN (2000) Structure of XynB, a highly thermostable β-1,4-xylanase from *Dictyoglomus thermophilum* Rt46B.1, at 1.8 Å resolution. Acta Crystallogr D 56:1367–1375

Morris DD, Gibbs MD, Chin CW, Koh MH, Wong KKY, Allison RW, Nelson PJ, Bergquist PL (1998) Cloning of the *xynB* gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on Kraft pulp. Appl Environ Microbiol 64:1759–1765

Pace CN (1990) Measuring and increasing protein stability. Trends Biotechnol 8:93–98

Pikkemaat MG, Linssen ABM, Berendsen HJC, Janssen DB (2002) Molecular dynamics simulations as a tool for improving protein stability. Protein Eng 15:185–192

Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13:101–131

Sandgren M, Shaw A, Ropp TH, Wu S, Bott R, Cameron AD, Ståhlberg J, Mitchinson C, Jones AT (2001) The X-ray crystal structure of the *Trichoderma reesei* family 12 endoglucanase 3, cel12A, at 1.9 Å resolution. J Mol Biol 308:295–310

Shibuya H, Kaneko S, Hayashi K (2000) Enhancement of the thermostability and hydrolytic activity of xylanase by random gene shuffling. Biochem J 349:651–656

Sung WL (2003) Xylanases with enhanced thermophilicity and alkalophilicity. Patent WO 03/046169

Tatu U, Murthy SK, Vithayathil PJ (1990) Role of a disulfide cross-link in the conformational stability of a thermostable xylanase. J Protein Chem 9:641–646

Tenkanen M, Puls J, Poutanen K (1992) Two major xylanases of *Trichoderma reesei*. Enzyme Microbiol Technol 14:566–574

Tidor B, Karplus M (1993) The contribution of cross-links to protein stability: a normal mode analysis of the configurational entropy of the native state. Proteins 15:71–79

Törrönen A, Rouvinen J (1997) Structural and functional properties of low molecular weight endo-1,4-β-xylanases. J Biotechnol 57:137–149

Turunen O, Etuaho K, Fenel F, Vehmaanperä J, Wu X, Rouvinen J, Leisola M (2001) A combination of weakly stabilizing mutations with a disulfide bridge in the α-helix region of *Trichoderma reesei* endo-1,4-β-xylanase II increases the thermal stability through synergism. J Biotechnol 88:37–46

Turunen O, Vuorio M, Fenel F, Leisola M (2002) Engineering of multiple arginines into the Ser/Thr surface of *Trichoderma reesei* endo-1,4-β-xylanase II increases the thermotolerance and shifts the pH optimum towards alkaline pH. Protein Eng 15:141–145

Van den Burg B, Vriend G, Veltman OR, Venema G, Eijsink VGH (1998) Engineering an enzyme to resist boiling. Proc Natl Acad Sci USA 95:2056–2060

Viikari L, Kantelinen A, Sundquist J, Linko M (1994) Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Rev 13:335–350

Vogl T, Brengelmann R, Hinz H-J, Scharf M, Lötzbeyer M, Engels JW (1995) Mechanism of protein stabilization by disulfide bridges: calorimetric unfolding studies on disulfide-deficient mutants of the α-amylase inhibitor Tendamistat. J Mol Biol 254:481–496

Wakarchuk WW, Sung WL, Campbell RL, Cunningham A, Watson DC, Yaguchi M (1994) Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. Protein Eng 7:1379–1386