The Use of Forced Protein Evolution to Investigate and Improve Stability of Family 10 Xylanases

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 52, Issue of December 24, pp. 54369–54379, 2004

Simon R. Andrews‡, Edward J. Taylor‡§, Gavin Pell‡, Florence Vincent‡, Valérie M.-A. Ducros§, Gideon J. Davies‡, Jeremy H. Lakey‡, and Harry J. Gilbert‡¶

From the §Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5YW and ‡Institute of Cell and Molecular Biosciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, United Kingdom

Metal ions such as calcium often play a key role in protein thermostability. The inclusion of metal ions in industrial processes is, however, problematic. Thus, the evolution of enzymes that display enhanced stability, which is not reliant on divalent metals, is an important biotechnological goal. Here we have used forced protein evolution to interrogate whether the stabilizing effect of calcium in an industrially relevant enzyme can be replaced with amino acid substitutions. Our study has focused on the GH10 xylanase CjXyn10A from Cellulovibrio japonicus, which contains an extended calcium binding loop that confers proteinase resistance and thermostability. Three rounds of error-prone PCR and selection identified a treble mutant, D262N/A80T/R347C, which in the absence of calcium is more thermostable than wild type CjXyn10A bound to the divalent metal. D262N influences the properties of the calcium binding site, A80T fills a cavity in the enzyme, increasing the number of hydrogen bonds and van der Waals interactions, and the R347C mutation introduces a disulfide bond that decreases the free energy of the unfolded enzyme. A derivative of CjXyn10A (CjCjXyn10A) in which the calcium binding loop has been replaced with a much shorter loop from Cellulomonas fimi CjXyn10A was also subjected to forced protein evolution to select for thermostabilizing mutations. Two amino acid substitutions within the introduced loop and the A80T mutation increased the thermostability of the enzyme. This study demonstrates how forced protein evolution can be used to introduce enhanced stability into industrially relevant enzymes while removing calcium as a major stability determinant.

Microorganisms utilize polymeric substrates by secreting hydrolytic enzymes into the extracellular milieu. This external environment is highly variable, and thus there is a strong selection pressure for secreted microbial enzymes to display stability to fluctuations in pH, temperature, or to proteinase attack. Analysis of the properties of related intra- and extracellular glycoside hydrolases demonstrates the stability of secreted glycoside hydrolases compared with their cytoplasmic or periplasmic counterparts (1). An elegant example of this variation in stability is found in the glycoside hydrolase family (GH) Xylanases expressed by the mesophilic bacteria Cellulovibrio japonicus and Cellulovibrio mixtus. C. japonicus expresses three GH10 xylanases; two are secreted into the extracellular environment (CjXyn10A and CjXyn10C), whereas CjXyn10D is translocated into the periplasm (2). C. mixtus also produces a periplasmic GH10 xylanase, CmXyn10B, which displays 90% sequence identity with CjXyn10D (1). Although recombinant CjXyn10A and CjXyn10C, in common with the other extracellular GH10 xylanases characterized to date, display thermostability and resistance to proteinase attack (3–6), purified CjXyn10B is thermolabile and subject to rapid proteinolytic inactivation (1).

In view of the close sequence similarity but significant difference in stability, CjXyn10A and CmXyn10B represent particularly useful models for interrogating the structural basis for stability in extracellular enzymes. Both enzymes display a typical (β/α)_{8} barrel with the substrate binding cleft running along the full-length of the enzymes (7, 8). CmXyn10B is the active GH10 xylanase described to date against both xylooligosaccharides and a range of xylans, whereas CjXyn10A displays properties that are typical of extracellular family 10 enzymes (8, 9). CjXyn10A displays resistance to proteinase attack and thermal inactivation at temperatures <65 °C in the presence of calcium; however, removal of the divalent metal ion causes a dramatic increase in thermo and proteolytic inactivation (6). Structural and site-directed mutagenesis studies of CjXyn10A show that calcium confers stability by binding to an extended loop (designated loop 7) between β-strand 7 and α-helix 7 (6). The metal ion makes co-ordinating bonds with the OD1 of Asp-262, OD1 and OD2 of Asp-256, OD1 of Asn-261, and the carbonyl groups of Asn-253 and Asn-258, with the octahedral geometry completed by a water molecule. The cal-

Received for publication, August 6, 2004, and in revised form, September 27, 2004 Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M409044200

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To whom correspondence should be addressed. Tel.: 44-(0)191-2227424; E-mail: h.j.gilbert@ncl.ac.uk.
cium binding site of CjXyn10A displays relatively low affinity ($K_d \sim 100 \mu M$) for the metal, and thus, it is unclear how the utilization of the metal ion as a major stability determinant confers a significant selective advantage.

Thermostability is an important phenotype of industrial enzymes. Not only does it decrease enzyme costs by extending the life of the biocatalysts, but many biotechnological processes, several of which utilize xylanases (e.g. paper pulp and animal feed sectors) operate at elevated temperatures (10, 11). Although calcium often plays a key role in the thermostability of enzymes, the inclusion of the metal ion is problematic as the formation of inorganic precipitates has deleterious effects on fermentation and downstream processing, whereas the accumulation of calcium salts in some products, such as beer, is not acceptable (12). Furthermore, in multienzyme industrial processes, exemplified by the biotransformation of starch into fructose, calcium may confer thermostability on some enzymes (amyloses) while inhibiting other biocatalysts (xylose/glucose isomerase) (13–16). Here we have used a directed-protein evolution approach to interrogate mechanisms by which random mutations can increase the stability of CjXyn10A and have evaluated the extent to which the information can be harnessed to develop generic principals for stabilization strategies of industrially relevant GH10 xylanases that do not rely on the presence of divalent metal ions.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmid Construction, and Growth Conditions**—The *Escherichia coli* strains TUNER:pLysS (Novagen, Madison, WI) and JM83 (17) were used in this study. The plasmid vectors used were pUC19 (18) and pET16b (Novagen). The recombinant plasmids were as follows. pNX1 encodes the catalytic domain of *C. japonicus* Xyn10A (residues Gly-1 to Arg-347), designated CjXyn10A, appended to the first 19 residues encoded by the multiple cloning region of pUC19 (9, 19); pNX2 encodes a derivative of CjXyn10A, designated CjCjXyn10A, in which the residues Asn-252 to Ser-272 of the calcium binding site of *E. coli* (20); pNX2 encodes a derivative of CjXyn10A, designated CjCjXyn10A, and the multiple cloning region of pUC19 (9, 19) was deleted prior to cloning into pET16b to give pNX3. MACRSTLED-RSP-CjXyn10A, which was used in crystallography experiments. The DNA encoding this enzyme was constructed by PCR using primers listed in Supplemental Table 1S and pNX1 as the template DNA. The amplified 1054-bp sequence, containing Neol and BamHI sites at the termini, was cloned into Neol/BamHI-restricted pET16b to generate pNX3. MACRSTLED-RSP-CjXyn10A comprises the catalytic domain of CjXyn10A (Gly-1 to Arg-347) containing an extended N-terminal sequence of MACRSTLED-RSP. The plasmid pNX4 encodes the mature form CjXyn10A (CmXyn10B) (8), whereas pCT1214 encodes the catalytic domain of *Clostridium thermocellum* Xyn10A (CjXyn10A), comprising residues 507–837 (20). The *E. coli* strains were cultured in Luria broth (LB) at 37 °C with aeration. The expression and subsequent purification of CjXyn10A and CjXyn10A (7), CjXyn10A (21), MACRSTLED-RSP-CjXyn10A, and CmXyn10B (8) were as described previously.

**Generation of Site-directed Mutants of CjXyn10A, CjCjXyn10A, CmXyn10A, and CmXyn10B**—Mutants were generated by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions using pNX1, pNX3, and pCT1214 as template DNA. The primers that were employed in the mutagenesis PCRs are shown in Table 1S. The complete sequence of DNA encoding the xylanase mutants were determined by MWG using M13 and T7 forward and reverse primers and appropriate custom sequencing primers.

**Construction and Screening of Error-prone PCR Libraries**—Each round of PCR comprised three successive error-prone PCRs using the following protocol. The PCRs contained 450 pM M13 forward and reverse primers, 3.9 mM MgCl2, 5 ng of template DNA, 220 μM dATP, 2.4 μM dCTP, 2.5 μM dGTP, and 2.5 μM of BioTaq DNA polymerase from *Thermus aquaticus*. The PCRs were made up to 100 μl, overlaid with 50 μl of mineral oil, and then incubated in a Technicon thermocycler at 94 °C for 3 min and then 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 4 min for 30 cycles followed by 10 min at 72 °C. The product generated from the first PCR was appropriately diluted and used as the template in the second PCR, and the product of the second PCR was used as the template in the third PCR. The final amplified product was digested with EcoRI and HindIII and cloned into similarly restricted pUC19, and the recombinant DNA molecules were transformed into *E. coli* SURE (Stratagene) by electroporation. The resultant colonies, comprising the error-prone PCR library, were plated onto LB-agar plates at a density of ~2000 colonies per plate and incubated for 16 h at 37 °C. The plates were then chilled at ~20 °C for 10 min, after which they were overlaid with 50 mM potassium phosphate-12 mM citrate buffer, pH 6.5, containing 0.8% agar and 0.5% Blue-Xylan (Megazyme International County Wicklow, Ireland) and incubated at the permissive temperature of 37 °C for 3 h. Residual xylanase activity was assessed by looking for clear haloes around the colonies within a blue background. Plasmid DNA was recovered from identified colonies using the Qiagen gel extraction and miniprep plasmid kits.

**Enzyme and Stability Assays**—The activity of CjXyn10A against 4-methylumbelliferyl-β-D-glucobioside (MUGase activity) was determined as described previously (6). Xylanase activity was performed essentially as described by Charnock et al. (9) except that the release of reducing sugar was determined using the Somogyi-Nelson reagent (22), and the substrate used was 0.2% glucuronoxylan. Each assay was performed in triplicate. The activity of CmXyn10B against 4-nitrophe- nyl-β-D-celllobioside was as described by Peli et al. (8). Thermo inacti- vation assays used the methods of Spaepen et al. (9). Individual activity was assayed by assaying MUCase activity. Differential scanning calorimetry (DSC) and isothermal titration calorimetry were carried out as described previously (6).

**Titration of Thiol Groups**—The number of free cysteine residues was determined by the method of Eillman et al. (23). Enzyme (60 μl) denatured in 100 mM Tris-HCl buffer, pH 7.5, containing 6.4 mM guanidine hydrochloride and 1 mM EDTA, was degassed under a vacuum. To both the protein and a blank (lacking the enzyme) was added 5.5′-dithiobis(2-nitrobenzoic acid), which had also been extensively degassed, to a final concentration of 1 mM, and the increase in A412 nm was determined. When 5.5′-dithio-bis(2-nitrobenzoic acid) reacts with a cysteine an equivalent amount of 5-thio-2-nitro-benzoic acid is released, which has a molar extinction coefficient of 13,700 M−1 cm−1.

**MALDI-TOF Mass Spectrometry and Trypsin Digest Peptide Mapping**—Wild type and mutants of CmXyn10B (1 mg/ml) were digested with trypsin (added to a final concentration of 20 μg/ml) in 100 mM sodium HEPES buffer, pH 6.8. Approximately 25 ng of the digested protein in water was subjected to MALDI-TOF analysis. The number and mass of the theoretical peptides generated by trypsin were determined using the ExPASy molecular biology server, Peptide Cutter tool (ca.expasy.org/tools/peptide-mass.html).

**Intermolecular Disulfide Assay Using SDS-PAGE**—Duplicated protein samples were loaded in 8 μl urea and 8 μl containing 1 M β-mercaptoethanol at 37 °C for 45 min. Bromphenol was added to a final concentration of 0.01%, the samples were electrophoresed on 10% (v/v) Bis-polyacrylamide gels, and proteins were visualized by staining with Coomassie Blue.

**Guanidinium Chloride Denaturation**—Circular dichroism (CD) spectroscopy was carried out using a Jasco J-810 spectropolarimeter. For far-UV CD spectra (260–220 nm) the bandwidth was set to 2 nm, scanning speed was set to 20 nm/min, and 0.2-mm-path length stopped cuvettes (Hellma) were used. The concentration of the proteins was 2.0 mg/ml in 10 mM sodium phosphate buffer, pH 7.0. All spectra shown are averages of five scans with the bandwidth set to 1 nm. Stock solutions of guanidine hydrochloride were determined by refractive index, and the dilutions were confirmed by weight. The ΔG of the folding step was determined by least squares analysis of the in-folding transition including the pre-transition and post-transition slopes (24).

**Crystallization and Data Collection**—Pure proteins, as judged by SDS-PAGE, were washed into water by repeated dilution (40 volumes of water) and concentration in a VIVASPIN 10-kDa concentrator. The wild type and mutant proteins of CjXyn10A were screened by the hanging drop vapor diffusion method using the PEG/Ion and Crystal screens (Hampton Research). Crystals grew in ~2–4 days at 18 °C. Drops contained 1 or 2 μl of protein (20 mg/ml) mixed with 1 μl of each concentration. Single crystals were obtained from 15% ethylene glycol before flash-freezing in boiling liquid nitrogen. Wild type MACRSTLED-RSP-CjXyn10A and the mutant D262N were crystallized in 0.2 M MgCl2, 5 mM CaCl2, 20% polyethylene glycol 3550, 15% ethylene glycol, whereas A80T/D262N/R347C was crystallized in 0.6 M Mn(NO3)2, 5 mM CaCl2, 15–25% polyethylene glycol 3550.

Data for native and mutant forms of CjXyn10A were collected at the European Synchrotron Radiation Facility beamline ID14-EH4 using a
Table 2S.

Cambridge, UK), and the data statistics are presented in Supplemental deposited at the Macromolecular Structures Data base (EBI, hood refinement. The coordinates and observed structure factors were

QUANTA (Accelrys) were interspersed with cycles of maximum-likelihood corrections of the model using the X-FIT routines of the program during maximum likelihood refinement using REFMAC (28). Manual

monitor various refinement strategies such as the weighting of geomet-

ically set aside for cross-validation analysis (27) and were used to

20–3.0 Å with an outer radius of Patterson integration of 25 Å, and the

CCP4 version of the program AMoRe. Data in the resolution range

and its derivatives were solved by molecular replacement using the

ADSC Quantum-4 charge-coupled device as detector. All data were

processed and scaled with the HKL suite (25) and all other computing

used the CCP4 suite (26) unless otherwise stated.

Structure Solution and Refinement—MACRSTLEDSP-CXyn10A

and its derivatives were solved by molecular replacement using the

CCP4 version of the program AmoRe. Data in the resolution range

20–3.0 Å with an outer radius of Patterson integration of 25 Å, and the

protein atoms of the catalytic core domain of CXyn10A (1clx.pdb) as the

search model. Before refinement, 5% of the observations were immedi-

ately set aside for cross-validation analysis (27) and were used to

monitor various refinement strategies such as the weighting of geomet-

tical and temperature factor restraint and the insertion of solvent water

during maximum likelihood refinement using REFMAC (28). Manual

corrections of the model using the X-FIT routines of the program

QUANTA (Accelrys) were interspersed with cycles of maximum-likeli-

hood refinement. The coordinates and observed structure factors were

deposited at the Macromolecular Structures Data base (EBI, Cambridge, UK), and the data statistics are presented in Supplemental Table 2S.

RESULTS DISCUSSION

Error-prone PCR of Wild Type CXyn10A—Error-prone PCR was used to introduce random mutations using a regime that generated libraries of xylanase mutants in which ~50% of the enzymes generated in the first round displayed catalytic activity at 37 °C (permissive temperature). Sequencing of 20 clones picked at random revealed an average mutation rate of three amino acid substitutions in the encoded xylanases. The frequency of mutations was as follows: AT → TA, 84%; GC → AT, 10%; AT → GC, 6%. The error-prone PCR library, comprising 100,000 clones, was screened for bacteria expressing thermostable xylanases in the absence of calcium. After incubation at 70 °C for 15 min (conditions that completely inactivate wild type CXyn10A), three colonies expressed a functional xylanase (Table I). The sequence of the genes encoding the thermostable xylanases all contained a nucleotide change in the codon for Asp-262 leading to the introduction of the D262N mutation. The observation that both codons for asparagine were evident in the selected colonies, one of which contained an additional silent mutation, indicate that the thermostable xylanases were derived from independent amplification events.

One of the xylanase clones containing the D262N mutation, designated d262n, was subjected to a second round of error-prone PCR, which generated ~100,000 colonies, with 30% displaying functional xylanase activity at the permissive temperature. The mutant library was again screened for clones that expressed a thermostable xylanase by incubating the colonies at 75 °C for 15 min, conditions that inactivate d262n. Three clones were shown to express a xylanase that retained activity after treatment at 75 °C. The sequence of cxyn10a in these mutants revealed two amino acid substitutions in the encoded xylanases; D262N/A80T or D262N/A80V (Table I). One of these clones (designated d262n/a80t) was subjected to a third round of error-prone PCR, and again, a library of around 105 clones was constructed, of which only 25% displayed xylanase activity at the permissive temperature of 37 °C. Screening the bacterial colonies at 78 °C for 120 min, which completely inactivates CXyn10A expressed by d262n/a80t, generated five clones that display xylanase activity at the non-permissive temperature. The five mutants, containing between one and three amino acid substitutions, in addition to D262N/A80T (mutations in the

### TABLE I

| Enzyme         | Round of PCR | Mutant name | Amino acid changes | Codon change |
|---------------|--------------|-------------|--------------------|--------------|
| CjXyn10A      | 1            | D262N       | D262N              | GAT262AAC    |
| CjXyn10A      | 1            | D262N       | D262N              | GAT262AAC    |
| CjXyn10A      | 1            | D262N/A69A  | D262N              | GAT262AAC    |
| CjXyn10A      | 2            | D262N/A80T  | A80T               | GCC80ACC     |
| CjXyn10A      | 2            | D262N/A80T  | A80T               | GCC80ACC     |
| CjXyn10A      | 2            | D262N/A80V  | A80V               | GCC80GC     |
| CjXyn10A      | 3            | D262N/A80T/R347C | R347C               | CGT347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/A98T | R347C, A98T       | CGT347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/Y145F/I160F | R347C, Y145F, I160F | CGT347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/Y145F/I160F | R347C, A224T       | GTC347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/Y145F/I160F | R347C, A224T       | GTC347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/G138S/I242V | R347C, G138S, I242V | GTC347TGT   |
| CjXyn10A      | 3            | D262N/A80T/R347C/G138S/I242V | R347C, G138S, I242V | GTC347TGT   |
| CjXyn10A      | 1            | C/D257N/P85S | cfD5N/P85S        | GAC/257AAC   |
| CjXyn10A      | 1            | C/D257N/P85S | cfD5N/P85S        | GAC/257AAC   |
| CjXyn10A      | 2            | C/P255S     | cfP255S           | GAC/257AAC   |
| CjXyn10A      | 2            | C/P255S     | cfP255S           | GAC/257AAC   |
| CjXyn10A      | 2            | C/P255S/A80T | cfP255S/A80T     | GAC/257AAC   |
| CjXyn10A      | 2            | C/P255S/A80V | cfP255S/A80V     | GAC/257AAC   |
| CjXyn10A      | 3            | C/P255S/A80V | cfP255S/A80V     | GAC/257AAC   |
| CjXyn10A      | 3            | C/P255S/A80V | cfP255S/A80V     | GAC/257AAC   |

*These mutants were used as the template to construct the following error-prone PCR library.*
of the D262N mutation into loop 7 increases the thermostability of wild type xylanase CjXyn10A, in which the extended calcium binding loop (residues Asn-252 to Ser-272 in CjXyn10A) was replaced with the much smaller, calcium-independent loop CjXyn10A, in which the extended calcium binding loop (residues Asn-252 to Ser-272 in CjXyn10A) was replaced with the much smaller, calcium-independent loop CjXyn10A, in which the extended calcium binding loop (residues Asn-252 to Ser-272 in CjXyn10A) was replaced with the much smaller, calcium-independent loop

Characterization of the Thermostable Mutants of CjXyn10A

CjXyn10A was shown previously to be less thermostable than wild type CjXyn10A, and CfCjXyn10A—D262N, A80T, R347C, D262N/A80T, and D262N/A80T/R347C, respectively (Table II). The DSC scans showed that the thermal inactivation of wild type CjXyn10A, A80T, or D262N caused similar increases in thermostability of wild type CjXyn10A (6). The gene encoding the CjXyn10A was subjected to three rounds of error-prone PCR to generate thermostable variants of the xylanase. The methodology, size of libraries, and selection strategy was the same as for CjXyn10A described above, except that the first-, second-, and third-round PCR libraries were screened at 60, 65, and 70 °C, respectively. Each round of PCR generated a dominant thermostabilizing locus (present in all thermostable clones). In the first round the mutation, which was located in the inserted loop from CjXyn10A, was either cfD257N or cfP255S, the second round mutation was either A80T or A80V, and the third round mutation was cfT254A, again within the CjXyn10A loop, Table I.

Characterization of the Thermostable Mutants of CjXyn10A and CfCjXyn10A—To confirm that the conserved mutations in CjXyn10A and CfCjXyn10A, generated in each round of error-prone PCR, conferred the observed thermostability, site-directed mutagenesis was used to introduce D262N, A80T, and R347C into wild type CjXyn10A in various combinations generating D262N, A80T, R347C, D262N/A80T, A80T/R347C, D262N/R347C, and D262N/A80T/R347C, whereas cfP255S, cfP255S/A80T, and cfP255S/A80T cfT254A were introduced into unmodified CfCjXyn10A. The enzymes produced by error-prone PCR and site-directed mutagenesis were purified to electrophoretic homogeneity, and their biophysical properties were assessed. The thermostability of D262N, D262N/A80T, D262N/A80T/R347C, cfP255S, cfP255S/A80T, and cfP255S/A80T cfT254A both with respect to loss of enzyme activity at the respective non-permissive temperatures and the melting temperature, determined by DSC, were indistinguishable from the enzymes isolated from the respective first-, second-, and third-round error-prone PCR libraries (data not shown). The pseudo-first order constants for thermal inactivation at 62 °C in the presence of 2 mM EDTA are 2.8 × 10^{-3}, 3.2 × 10^{-3}, and 2.2 × 10^{-3} s^{-1} for wild type CjXyn10A, D262N, and D262N/A80T, respectively (Table II and Fig. 1), and the melting temperatures (Table III) for these enzymes are 62.0 °C (wild type), 67.5 °C (D262N), 69.0 °C (D262N/A80T), and 72.5 °C (D262N/A80T/R347C), respectively. The melting temperatures for wild type CjXyn10A, cfP255S, cfP255S/A80T, and cfP255S/A80T cfT254A are 56.2, 59.3, 61.3, and 64.3 °C (Table III), and the pseudo-first order rate constants for thermal inactivation at 55 °C in the presence of EDTA are 2.6 × 10^{-3}, 2.6 × 10^{-4}, and 2.0 × 10^{-3} s^{-1}, respectively (Table II). The DSC scans showed that the thermal unfolding of each mutant is irreversible; it was not possible to obtain a refolding scan, and there was evident precipitation of the proteins. The introduction of the A80T mutation into wild type CjXyn10A or D262N caused similar increases in thermostability, indicating that this amino acid substitution acts in isolation of the D262N amino acid substitution (Table II). By contrast, the R347C mutant did not enhance the thermostability of wild type CjXyn10A, A80T, or D262N (Table II). R347C, A80T/R347C, and D262N/R347C contain an additional thiol that could be titrated with 5,5′-dithio-bis-2-nitrobenzoic acid, indicating that in these mutants Cys-347 does not form a disulfide bridge (Table IV). It would appear, therefore, that the consecutive introduction of the mutations D262N, A80T, and

| Enzyme | Mutant | Ca^{2+}/EDTA | Thermo k_{1/2}^{a} |
|--------|--------|--------------|------------------|
| CjXyn10A | Wild type | Ca^{2+} | 9.6 × 10^{-4} |
| CjXyn10A | Wild type | EDTA | 2.8 × 10^{-2} |
| CjXyn10A | D262N | Ca^{2+} | 2.1 × 10^{-5} |
| CjXyn10A | EDTA | 2.2 × 10^{-3} |
| CjXyn10A | A80T | EDTA | 7.9 × 10^{-3} |
| CjXyn10A | A80T | Ca^{2+} | 2.8 × 10^{-4} |
| CjXyn10A | R347A | EDTA | 3.4 × 10^{-2} |
| CjXyn10A | A80T/R347C | Ca^{2+} | 3.2 × 10^{-4} |
| CjXyn10A | D262N/R347C | EDTA | 2.8 × 10^{-3} |
| CjXyn10A | D262N/A80T | EDTA | 3.2 × 10^{-4} |
| CjXyn10A | D262N/A80T/R347C | EDTA | 1.2 × 10^{-4} |
| CjXyn10A | D262N/A80T/R347C/GalC7A | EDTA | 4.0 × 10^{-4} |
| CjXyn10A | Wild type | EDTA | 2.6 × 10^{-5} |
| CjXyn10A | P255S | EDTA | 2.6 × 10^{-5} |
| CjXyn10A | P255S/A80T | EDTA | 8.1 × 10^{-5} |
| CjXyn10A | P255S/A80T cfT254A | EDTA | 2.0 × 10^{-5} |
| CmXyn10B | Wild type | EDTA | 9.5 × 10^{-4} |
| CmXyn10B | L375C | 2.8 × 10^{-3} |
| CmXyn10B | L375C/T34C | 3.7 × 10^{-3} |
| CmXyn10B | L375C/G35C | 3.5 × 10^{-3} |
| CmXyn10B | L375C/L36C | 1.1 × 10^{-2} |
| CmXyn10B | L380C | 2.5 × 10^{-5} |
| CmXyn10B | L380C/A26C | 2.0 × 10^{-5} |
| CmXyn10B | L380C/A26C | 2.1 × 10^{-5} |
| CmXyn10B | Wild type | 1.2 × 10^{-3} |
| CmXyn10A | Y387C | 2.9 × 10^{-3} |
| CmXyn10A | Y387C/Ntc | 2.7 × 10^{-4} |

a The values in parentheses are the temperatures in °C at which enzyme inactivation was assessed.

b Nt, a cysteine has been inserted into the N-terminal sequence.

cf The values in parentheses are the temperatures in °C at which enzyme inactivation was assessed.

Ca^{2+}/EDTA was added at a concentration of 2 mM.

Calcium or EDTA was added at a concentration of 2 mM.
activity reveals the proportion of enzyme molecules that have undergone irreversible denaturation. The temperatures at which the CfXyn10A derivatives lose 50% of their activity are as follows: wild type (with EDTA), 57 °C; wild type (with Ca²⁺), 62 °C; D262N, 60 °C; D262N/A80T, 64 °C; D262N/A80T/R347C, 67 °C. The corresponding values for CfXyn10A-derived enzymes are: wild type, 52 °C; cfP255N, 56 °C; cfP255N/A80T, 58 °C; cfP255N/A80T/T254A, 62 °C, Fig. 2. These results are entirely consistent with the enhanced stability at the non-permissive temperatures of 62 °C for the CfXyn10A mutants, 55 °C for the CfCfXyn10A mutants and the increase in melting temperature displayed by the mutants.

**Influence of Calcium on Thermostability**—To investigate whether the enhanced thermostability displayed by the mutants was calcium-independent, the rate of inactivation of the enzymes in the presence of 2 mM calcium or EDTA was compared. Although the divalent metal ion significantly enhanced the stability of wild type CfXyn10A and A80T, it had no effect on any of the other CfXyn10A mutants or CfCfXyn10A (Table II). To assess whether D262N bound calcium, the enzyme was treated with Chelex-100 to remove any bound divalent metal ions and then titrated with calcium. Isothermal titration calorimetry revealed that D262N displays an affinity for the divalent ion that is 100-fold lower than the wild type xylanase (data not shown). Thus, the D262N mutation confers calcium-independent thermostabilization, a property that is of industrial relevance.

**Properties of the CfXyn10A Mutants**—The catalytic activity of all the CfXyn10A and CfCfXyn10A mutants (Table V) are not significantly different from the cognate wild type xylanase, demonstrating that the thermostabilizing mutations do not influence the topology of the active site. Wild type CfXyn10A displayed reduced activity in several organic solvents, with dimethylformamide causing the largest decrease in hydrolytic activity revealing the proportion of enzyme molecules that have undergone irreversible denaturation. The temperatures at which the CfXyn10A derivatives lose 50% of their activity are as follows: wild type (with EDTA), 57 °C; wild type (with Ca²⁺), 62 °C; D262N, 60 °C; D262N/A80T, 64 °C; D262N/A80T/R347C, 67 °C. The corresponding values for CfCfXyn10A-derived enzymes are: wild type, 52 °C; cfP255N, 56 °C; cfP255N/A80T, 58 °C; cfP255N/A80T/T254A, 62 °C, Fig. 2. These results are entirely consistent with the enhanced stability at the non-permissive temperatures of 62 °C for the CfXyn10A mutants, 55 °C for the CfCfXyn10A mutants and the increase in melting temperature displayed by the mutants.

**Table IV**

| Enzyme      | Mutant                  | Ca²⁺/EDTA | Tₘ  |
|-------------|-------------------------|-----------|-----|
| CfXyn10A    | Wild type               | Ca²⁺      | 66.5|
| CfXyn10A    | Wild type               | EDTA      | 61.2|
| CfXyn10A    | D262N                   | EDTA      | 65.2|
| CfXyn10A    | D262N/A80T              | EDTA      | 69.5|
| CfXyn10A    | D262N/A80T/R347C        | EDTA      | 72.2|
| cfCfXyn10A  | Wild type               | EDTA      | 56.2|
| cfCfXyn10A  | cfP255S                 | EDTA      | 59.3|
| cfCfXyn10A  | cfP255S/A80T            | EDTA      | 60.3|
| cfCfXyn10A  | cfP255S/A80T/T254A      | EDTA      | 64.1|
| CmXyn10B    | Wild type               | EDTA      | 58.6|
| CmXyn10B    | L375C                   | EDTA      | 55.0|
| CmXyn10B    | L375C/T34C              | EDTA      | 54.5|
| CmXyn10B    | L375C/G35C              | EDTA      | 53.6|
| CmXyn10B    | L375C/L36C              | EDTA      | 43.2|
| CmXyn10B    | L380C                   | EDTA      | 57.0|
| CmXyn10B    | L380C/A26C              | EDTA      | 62.9|
| CmXyn10B    | L380C/A30C              | EDTA      | 62.7|

* EDTA or calcium was added at a concentration of 2 mM.

R347C into CfXyn10A and cfP255S, A80T, and cfT254A into CfCfXyn10A conferred the enhanced thermostability displayed by the enzymes isolated from the mutagenesis program. Thus, the mutants introduced into wild type CfXyn10A and CfCfXyn10A by site-directed mutagenesis were used in subsequent experiments.
Xyn10A loop into CjXyn10A greatly increased the sensitivity of the xylanase to proteinase inactivation. Although the introduction of the treble mutation cfP255S/A80T/cfT254A increased the stability of CjCjXyn10A 2-fold, the enzyme was still 20-fold more sensitive to chymotrypsin than wild type CjXyn10A in the absence of calcium.

It could be argued that the flexible nature of the extended N-terminal sequence in CjXyn10A may speed up the denatur-
or2m EDTA. At regular time intervals aliquots were removed, chymotrypsin was inactivated, and residual MUCase activity remaining was determined.

The hypothesis the thermostability of a derivative of CjXyn10A displays the same thermostable properties as CjXyn10A, and the introduction of the D262N and A80T mutations into either CjXyn10A or mCjXyn10A afforded similar increases in thermostabilization (data not shown).

Analysis of the Mechanism of R347C Thermostabilization—The R347C mutation may cause protein stabilization by the formation of a disulfide bond. Inspection of the primary sequence of CjXyn10A revealed two cysteines at positions 269 and 273 (of the catalytic domain) that form a disulfide bond (7). The gene encoding CjXyn10A, however, was inserted into pUC19 in-frame with the vector translational start codon of lacZ. Thus, the 19 N-terminal residues of CjXyn10A are encoded by the multiple cloning region of pUC19 (these vector-encoded residues are prefixed by gal)-18), which contains a cysteine at position 10 (gal-Cys-10; See Fig. 3). To investigate whether Cys-347 makes a disulfide bond with gal-Cys-10, the mutation gal-C10A was introduced into D262N/A80T/R347C, and the resultant mutant was shown to display the same thermostability as D262N/A80T/R347C. By contrast, when the C-terminal cysteine mutation was inserted in the full-length xylanase. The residues in Table VI.

Cysteines were also inserted into the extremities of the ordered region of CmXyn10B (as judged by x-ray crystallography), which would be in close proximity in the folded protein and, thus, capable of forming disulfide bonds (L375C/T34C, L375C/G35C, and L375C/L36C). A further two sets of mutations were designed, L380C/A26C and L380C/A30C, in which two different N-terminal cysteines were introduced into the region of CmXyn10B before Gly-35, the first ordered amino acid, and the thiol-containing amino acid was substituted for Leu-380, the last ordered residue seen in the crystal structure. The activity of the mutant enzymes is similar to wild type CmXyn10B against 4-O-methyl-D-glucuronoxylan and 4-nitrophenoxy-β-D-cellulobiose, verifying that these mutations, as predicted by their location on the opposite side of the protein from the active site, had no significant effect on catalytic activity (Table V).

The thermostability of the wild type and mutant enzymes was initially characterized using a kinetic assay before and after treatment at 61 or 55 °C. The data (Table II) show that the three double mutations introduced into the ordered region of the crystal structure (L375C/T34C, L375C/G35C, and L375C/L36C) destabilized the protein. By contrast, at 62 °C L380C/A26C and L380C/A30C are ~8-fold more stable than the wild type enzyme, whereas the mutation L380C alone caused a modest decrease in thermostability.

DSC was used to directly measure the thermal denaturation of the mutant proteins. The data reveal irreversible thermal unfolding of the wild type xylanase and its mutant derivatives as evidenced by the aggregation and precipitation of the protein, the sharp decline in the trace at the end of each DSC scan, and the inability to measure a refolding scan (data not shown), preventing a rigorous thermodynamic analysis of protein unfolding. The midpoint of the thermal unfolding (Tm) increased by 4.5 °C for both L380C/A26C and L380C/A30C, compared to wild type CmXyn10B.

**Table VI**

| Proteinase | Mutant        | Ca\(^2+\)/EDTA | Proteolysis of CjXyn10A |
|------------|---------------|----------------|------------------------|
|            | CjXyn10A      |                |                        |
| Wild type  | CjXyn10A      | 1.3            |                        |
| Wild type  | CjXyn10A      | 1.9            |                        |
| Wild type  | CjXyn10A      | 2.4 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 1.1 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 1.3 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 9.5 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 7.7 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 5.7 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 2.2 \times 10^{-5} |                        |
| Wild type  | CjXyn10A      | 1.6 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 2.4 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 1.1 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 1.3 \times 10^{-3} |                        |
| Wild type  | CjXyn10A      | 9.5 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 7.7 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 5.7 \times 10^{-4} |                        |
| Wild type  | CjXyn10A      | 2.2 \times 10^{-5} |                        |

**Fig. 3. Schematic of the CjXyn10 derivatives.** The capital letter numbered residues are in the original catalytic domain of CjXyn10A (G1 is the first enzyme in the catalytic domain and corresponds to Gly-265 in the full-length xylanase). The residues in lowercase are as follows. Gal residues (prefixed by gal- in the text) are encoded by the multiple cloning region of pUC19 (cf from the text) are from CjXyn10A and are inserted between amino acids Asn-248 and Ser-278 of CjXyn10A to generate CjXyn10A.

Cysteines were also inserted into the extremities of the ordered region of CmXyn10B (as judged by x-ray crystallography), which would be in close proximity in the folded protein and, thus, capable of forming disulfide bonds (L375C/T34C, L375C/G35C, and L375C/L36C). A further two sets of mutations were designed, L380C/A26C and L380C/A30C, in which two different N-terminal cysteines were introduced into the region of CmXyn10B before Gly-35, the first ordered amino acid, and the thiol-containing amino acid was substituted for Leu-380, the last ordered residue seen in the crystal structure. The activity of the mutant enzymes is similar to wild type CmXyn10B against 4-O-methyl-D-glucuronoxylan and 4-nitrophenoxy-β-D-cellulobiose, verifying that these mutations, as predicted by their location on the opposite side of the protein from the active site, had no significant effect on catalytic activity (Table V). The thermostability of the wild type and mutant enzymes was initially characterized using a kinetic assay before and after treatment at 61 or 55 °C. The data (Table II) show that the three double mutations introduced into the region of the crystal structure (L375C/T34C, L375C/G35C, and L375C/L36C) destabilized the protein. By contrast, at 62 °C L380C/A26C and L380C/A30C are ~8-fold more stable than the wild type enzyme, whereas the mutation L380C alone caused a modest decrease in thermostability.
with the wild type xylanase CmXyn10B (Table III). The most likely explanation for the increased thermostability is through either an inter- or intrachain disulfide bond. SDS-PAGE (Fig. 4) shows that ~65% of L380C dimerizes, as the predominant species is 84 kDa, twice the molecular mass of the protein treated with β-mercaptoethanol. The other mutants and wild type CmXyn10B have a molecular mass of ~42 kDa and are, thus, monomeric. To investigate whether L380C/A30C contains an intrachain disulfide bond, the mutant and the wild type xylanase were subjected to trypsin digestion and then MALDI-TOF mass spectrometry. The mass spectrometry peptide fingerprint (Fig. 5) revealed that L380C/A30C contained a fragment that was not present in the wild type CmXyn10B fingerprint, and its mass corresponded to the sum of the two peptides containing the engineered cysteines less the mass of two hydrogens, which are lost as the covalent disulfide bond is formed. To confirm interchain disulfide bond formation in L380C/A30C and L380C/A30C, the free sulfhydryl concentrations of the guanidinium-unfolded proteins were determined. The data show (Table IV) that wild type CmXyn10B and the mutants L380C/A26C and L380C/A30C contain one free sulfhydryl (i.e. cysteines not engaging in covalent interactions), whereas L380C has a value of 1.45, consistent with the partial dimerization of the mutant (65% dimer formation, Fig. 4). The cumulative data support the view that the introduced cysteines in the disordered regions of the enzyme generate disulfide bonds between the N and C terminus, and this interaction increases the thermostability of the enzyme.

Free Energy of Folded and Unfolded CjXyn10A—Since heat denaturation was irreversible, equilibrium denaturation measurements using guanidine hydrochloride (GdmCl) as a denaturant were performed to obtain the difference between the free energies of the folded and unfolded proteins at 25 °C. The normalized ellipticity values as a function of GdmCl concentration are shown in Fig. 6. Using the approach described by Pace (30), the midpoints of the GdmCl denaturation curves and the ΔG values for GdmCl-induced unfolding were determined. The concentration of GdmCl in which 50% of wild type CjXyn10A, D262N, D262N/A80T, and D262N/A80T/R347C are unfolded (U50) correspond to 1.11, 1.32, 1.55, and 1.75 M, respectively (Table VII), which is entirely consistent with the thermostability displayed by these proteins. By extrapolating the plot of unfolded protein versus denaturant concentration to a value of 0 M GdmCl, the free energy of folding from the denatured state in zero denaturant was calculated (ΔG_H2O). Although the ΔG_H2O for D262N mirrored its respective thermostability, this was not so for A80T/D262N and D262N/A80T/R347C (Fig. 6 and Table VII). The double mutant has a similar ΔG_H2O to the wild type enzyme, but the value for the triple mutant was half that of the wild type xylanase. The denaturation of this protein with its oxidized disulfide bridge gave a ΔG_H2O of 5.03 kcal mol⁻¹ compared with 10.10 kcal mol⁻¹ for the wild type enzyme. Since these mutant proteins show the highest U50 values, this is apparently contradictory. This behavior is due to a low m value, which measures the loss of ΔGm denaturant concentration and can be interpreted as a measure of the affinity of the proteins for the denaturant. For the triple mutant this is likely due to the inability to reach the fully unfolded form because the N and C termini are fixed. There is a lower free energy difference between the two states because the unfolded state is not truly unfolded and conformational entropy not fully explored. The effect of the double mutant is much less but may also indicate residual structure in the “denatured state” as has been found for several proteins (31). Since entropic effects are accepted for disulfide mutants it may also be true that the thermal stability of the folded state can be increased by mutations that create more native unfolded forms without covalent links.

Crystal Structure of CjXyn10A Derivatives—The crystal structure of wild type MACRSTLEDRSF-CjXyn10A and the mutants D262N and D262N/A80T/R347C were solved to 1.45, 1.20, and 1.50 Å, respectively, Crystals were only obtained in the presence of calcium, whereas Cj/CjXyn10A and its derivatives failed to crystallize. Surprisingly, despite D262N/A80T/R347C displaying very weak affinity for calcium, the metal is evident in the protein (resulting from the high concentration in the crystallization conditions). The co-ordination of the bound metal is identical in the two proteins except that the OD1 of Asp-262 is replaced by the OD1 of A80T in the mutant enzyme. Presumably this single difference is responsible for a >100-fold decrease in affinity of the xylanase for calcium. Although the amide carbonyl of Asn-262 can co-ordinate with calcium, the energy of this coordinate bond is considerably lower than the electrostatic interaction between the metal ion and the negative charge of the carboxylate of Asp-262. Because the thermostabilization of D262N is Ca²⁺-independent, we are unable to provide definitive insight into the structural basis of the enhanced stability displayed by the mutant. One may suggest that in the wild type xylanase the absence of calcium would result in significant electronic repulsion of the carboxylate side chains of Asp-262 and Asp-256, which would destabilize the loop and, thus, reduce the thermostability of the en-
zyme. The D262N would remove these repulsive effects, and perhaps in the absence of Ca\textsuperscript{2+}/H\textsubscript{11001} the amide nitrogen takes the place of the calcium itself in coordinating the other residues.

The crystal structure of A80T/D262N/R347C (Fig. 7) shows that the bulkier side chain of threonine fills the space, estimated at 15 Å\textsuperscript{3} in the wild type xylanase, which is not occupied by the smaller methyl group of Ala-80 in the wild type enzyme and makes numerous van der Waals contacts with surrounding residues such as Leu-81, Trp-83, His-79, Asp-123, and Asn-126. Furthermore, the hydroxyl group of the side chain of threonine makes a hydrogen bond with the backbone carbonyl of Asp-123. Support for the stabilizing effect of the A80T mutation is evident from comparison of GH10 xylanases. Of the 25 most closely related sequences to CjXyn10A, 22 have threonine in the equivalent position to Ala-80 in the Cellvibrio xylanase. It should be noted that the A80V mutation conferred a stabilizing effect that was similar to the A80T substitution. Although valine would not make an extra hydrogen bond with the backbone carbonyl, the bulky aliphatic side chain of the amino acid would be likely to make more extensive van der Waals interactions than the methyl group of Ala-80, leading to increased stability of the protein.

The crystal structure of D262N/A80T/R347C revealed ordered residues from gal-Pro-12 (the residue preceding Gly-1, the first amino acid of the catalytic domain) to the backbone atoms of Cys-347. The N-terminal 11 residues (MACRSTLEDRSP) and the side chain of the C-terminal cysteine were not visible in the crystal structure, indicating that these regions of the protein are disordered. It would appear, therefore, that the disulfide bond between Cys-347 and the cysteine in the extended N-terminal sequence MACRSTLEDRSP is within a region of the protein that does not contribute to the crystal structure of the enzyme.

**DISCUSSION**

In this study all the mutations isolated from each round of mutagenesis contained changes at the same amino acid locus. Although this may indicate that the protein contains a single clearly identified "weak" spot that is particularly prone to unfolding, the selection strategy, which employs a plate assay, identifies the thermostabilizing mutations that have the largest effect without compromising enzyme activity. Other thermostabilizing mutants may not have been identified because they cause a less dramatic increase in stability (than the mutants selected) and/or reduce catalytic activity. Indeed this is consistent with the observation that the A80T mutation was only identified in the second round of mutagenesis, as the thermostabilizing effect of this amino acid substitution is less than D262N. Furthermore, the library generated does not sample all the possible amino acid substitutions; any amino acid mutation requiring three or even two nucleotide changes to the cognate codon will be greatly underrepresented in the library. Thus, we have analyzed the effect on thermostability of only a proportion of the total single amino acid changes possible.
Furthermore, our approach is unlikely to reveal synergistic interactions between amino acid changes in which the individual mutations, in isolation, do not enhance thermostability. We did recombine (shuffle) the first round PCR library, and the two most thermostable mutants isolated from a screen of 20,000 clones contained the D262N/A80T/R347C mutation (data not shown), suggesting that our strategy has identified the most stable mutants in the library. It should be emphasized, however, that mutations, which require nucleotide change to a specific codon, that confer thermostability will be infrequently isolated through error-prone PCR, and thus, it is probable that amino acid substitutions other than D262N, A80T, and R347C will improve the stability of the xylanase.

The introduction of stable covalent disulfide bonds increases the rigidity of the unfolded molecule, and thus, the entropy of the unfolded peptide chain is reduced as reported previously (30, 32). Some of these studies, exemplified by the work of Pace et al. (30), show that the predicted increase in $\Delta G$ of the folded protein is greater than the thermostabilizing effect observed, and it was proposed that this difference is due to unfavorable enthalpic non-covalent interactions of the introduced cysteine side chains. These enthalpically unfavorable interactions can be observed in CmXyn10B and CtXyn10A, where the small decrease in stability of L380C and Y387C, respectively, are likely to reduce the overall beneficial effect on thermostability of the disulfide bond in the mutants L380C/A26C, and L380C/A30C in CmXyn10B and Y387C in CtXyn10A through a significant reduction in the free energy of the unfolded protein at elevated temperatures.

The increase in thermostability is not mirrored by corresponding improvements in resistance to proteolysis and organic solvents. C. japosicus is a mesophilic bacterium isolated from Japanese soil (33). It is unlikely that the temperature of the soil will increase above 40 °C, and thus, the capacity of calcium to confer thermostability is probably a bystander effect rather than a major evolutionary driver. Because the extracellular environment of microbial ecosystems displays high protease activity (3), resistance to proteolytic degradation exerts a strong selection pressure on the evolution of extracellular enzymes such as xylanases. This view is consistent with the observation that extracellular xylanases from both mesophilic and thermophilic hosts are resistant to proteinases (3, 5), whereas intracellular forms of these enzymes are susceptible to proteolytic attack (1). It is interesting to note that only three amino acid substitutions generate an enzyme that is more thermostable than the wild type xylanase, and thus, the dependence on calcium for this phenotype can easily be selected out. It is likely that the introduction of proteinase resistance requires more complex changes to CjXyn10A than a small number of amino acid changes, and thus, there is a strong evolutionary selection for the retention of the calcium binding loop.

To conclude, these studies demonstrate that forced protein evolution can be used to generate enzymes that display im-

![Fig. 7. Crystal structure of the thermostable mutants of CjXyn10A. Panel a shows orthogonal views of CjXyn10A with the three sites of improved stability indicated; the calcium binding loop, Ala-80, and the N- and C-terminal “contact” region where an inserted S-S bridge contributes to stability. Panel b reveals the structural basis of the A80T mutation. The introduced threonine both fills a void and, thus, makes van der Waals contacts with surrounding residues and allows a favorable hydrogen bond (dotted yellow line) with Asp-123. This figure was drawn with PyMol (DeLano Scientific, San Carlos, CA) and is in divergent (“wall-eyed”) stereo.](http://www.jbc.org/content/383/14/54378/F7.large.jpg)
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proved thermostability though the introduction of only three amino acid substitutions. Furthermore, the engineered enzyme displays enhanced thermostability independent of bound calcium, whereas the metal ion plays a key role in stabilizing the wild type enzyme. The study also demonstrates the introduction of a disulfide bond between and N and C terminus as a generic approach to improving the thermostability of GH10 xylanases. This study not only provides insight into the evolutionary drivers that have led to the stabilizing role of calcium in CjXyn10A but also shows how the industrial utility of enzymes can be radically improved through the introduction of metal ion-independent stability.

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The Use of Forced Protein Evolution to Investigate and Improve Stability of Family
10 Xylanases: THE PRODUCTION OF Ca2+-INDEPENDENT STABLE
XYLANASES

Simon R. Andrews, Edward J. Taylor, Gavin Pell, Florence Vincent, Valérie M.-A.
Ducros, Gideon J. Davies, Jeremy H. Lakey and Harry J. Gilbert

J. Biol. Chem. 2004, 279:54369-54379.
doi: 10.1074/jbc.M409044200 originally published online September 27, 2004

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