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ORIGINAL RESEARCH PAPER

C-Terminal carbohydrate-binding module 9_2 fused to the *N*-terminus of GH11 xylanase from *Aspergillus niger*

Wenxuan Xu · Yajuan Liu · Yanxin Ye · Meng Liu · Laichuang Han · Andong Song · Liangwei Liu

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Abstract
Objective The 9_2 carbohydrate-binding module (C2) locates natively at the *C*-terminus of the GH10 thermophilic xylanase from *Thermotoga marimita*. When fused to the *C*-terminus, C2 improved thermostability of a GH11 xylanase (Xyn) from *Aspergillus niger*. However, a question is whether the *C*-terminal C2 would have a thermostabilizing effect when fused to the *N*-terminus of a catalytic module.
Results A chimeric enzyme, C2-Xyn, was created by step-extension PCR, cloned in pET21a(+), and expressed in *E. coli* BL21(DE3). The C2-Xyn exhibited a 2 °C higher optimal temperature, a 2.8-fold longer thermostability, and a 4.5-fold higher catalytic efficiency on beechwood xylan than the Xyn. The C2-Xyn exhibited a similar affinity for binding to beechwood xylan and a higher affinity for oat-spelt xylan than Xyn.
Conclusion C2 is a thermostabilizing carbohydrate-binding module and provides a model of fusion at an enzymatic terminus inconsistent with the modular natural terminal location.

Keywords *Aspergillus niger* · Carbohydrate-binding module 9_2 · *N*-terminal fusion · Xylanase

Introduction

Hydrolases play a pivotal role in carbohydrate recyclization and renewable energy production. A hydrolase usually contains a carbohydrate-binding module (CBM) and 69 families of CBMs have been isolated (Carvalho et al. 2015). Xylanase (EC 3.2.1.8) is a hydrolase that breaks down the β-1,4-xylan backbone of a polysaccharide. It is widely used in food, feed, paper and pulp industry (van Gool et al. 2013; Zhao et al. 2013). An *Aspergillus niger* xylanase (Xyn) is a typical of family GH11 xylanase (GenBank: EU375728) (Chen et al. 2006), which usually contains no CBM. To fulfil biotechnological demands, the thermostability of Xyn needs to be improved because its thermal inactivation half-life ($t_{1/2}$) is only 21 min at 50 °C. To fuse a thermophilic CBM is a straightforward rational engineering strategy.

A thermophilic family 9 CBM (CBM9) locates natively at the *C*-terminus of a multimodular family

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W. Xu · Y. Liu · Y. Ye · M. Liu · L. Han · A. Song · L. Liu (✉)
From the Life Science College, Henan Agricultural University, 95 Wenhua Road, Zhengzhou 450002, Henan, People's Republic of China
e-mail: liangwei@henau.edu.cn

L. Liu
Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Zhengzhou, People's Republic of China

GH10 xylanase 10A from *Thermotoga marimita*. The CBM9 was first recognized as a cellulose-binding domain for binding to insoluble microcrystalline cellulose and was later identified as a xylan-binding module (Meissner et al. 2000; Notenboom et al. 2001; Winterhalter et al. 1995). When fused to the *C*-terminus of Xyn, the CBM9 caused an improvement of 2 °C in enzyme optimal temperature (T_{opt}) (Liu et al. 2011). The CBM9 contains two smaller modules, CBM9_1 (C1) and CBM9_2 (C2). When fused to the *C*-terminus, both the modules C1 and C2 improved the Xyn catalytic activity, and C2 additionally improved the enzyme's thermostability (Liu et al. 2012b).

Which terminus to fuse is the first consideration in module fusion. Presently, the terminus is intuitively selected consisting with a CBM natural terminal location (Furtado et al. 2015; Li and Shao 2006; Liu et al. 2011, 2012b; Mai-Gisondi et al. 2015; Ye et al. 2011). A puzzle is whether the *C*-terminal C2 would have a thermostabilizing effect when fused to the *N*-terminus of an enzyme. Only when the C2 also improves enzyme thermostability at the *N*-terminus, can it be regarded as thermostabilizing. A thermostabilizing CBM is more attractive than those only binding substrate (Furtado et al. 2015; Khan et al. 2013; Kittur et al. 2003; Mai-Gisondi et al. 2015; Mangala et al. 2003). To elucidate this puzzle, the C2 is fused to the Xyn *N*-terminus through a natural 29 amino acid linker peptide selected from the *T. marimita* xylanase 10A because it had been successfully used in module fusion (Liu et al. 2011, 2012a, b). Besides engineering the Xyn properties and elucidating the C2 functions, the study also provides a model of fusing a CBM at another enzyme terminus inconsistent with its natural terminal location.

Materials and methods

Materials and regents

The C2-Xyn was created using the following primers synthesized by Genewiz Inc (Beijing, China): P1 (GAAGGAGATATA*CATATG*ATGGTAGCGACAG CAAAAT)/P2 (AGGACCTCAGGCTTGATGAGCC TGAGGTTAC)/P3 (GTAACCTCAGGCTCATCAA GCCTGAGGTCCT)/P4 (GTG GTGGTG*CTCGAG*A GAGGAGATC). The primers P2 and P3 had a homologous sequence to allow the related PCR products to combine with each other in an overlap extension process. For cloning into pET21a(+) (Novagen), the primers P1 and P4 respectively had restriction sites (shown in italics) digested by *Nde*I and *Xho*I (Takara). *Ex*Taq DNA polymerase (Takara) was used to ensure DNA sequences correctly amplified.

Construction of the C2-Xyn

The C2 DNA fragment was amplified in 50 µl containing 1 µM primers P1/P2, 54 ng pET20b-Xyn-C2 template (Liu et al. 2012b), 1 U *Ex*Taq DNA polymerase, 4 µmol of dNTPs, and polymerase buffer. The PCR procedure was: pre-denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 64.9 °C for 1 min, and extension at 72 °C for 1 min. Containing the linker peptide sequence, the Xyn DNA fragment was amplified in 50 µl containing 1 µM P3/P4 primers, 49 ng pET20b-Glu-Xyn template (Liu et al. 2012a), 1 U *Ex*Taq DNA polymerase, 4 µmol of dNTPs, and polymerase buffer. The PCR procedure was: pre-denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 61.9 °C for 1 min, and extension at 72 °C for 1 min. The fragments C2 and Xyn were purified with a DNA extraction and clean kit (Qiagen).

The C2-Xyn DNA was created in an overlap extension PCR. A 50 µl mixture contained 1 µM primers P1/P4, 49.8 ng C2 and 68.4 ng Xyn fragments, 1 U *Ex*Taq DNA polymerase, 4 µmol dNTPs, and polymerase buffer. The PCR procedure was: five cycles of self-extension by annealing at 68.3 °C for 1 min, and then 22 cycles of annealing at 63.8 °C for 1 min and extension at 72 °C for 1.5 min. The products were purified with a DNA extraction and clean kit. After being digested with *Nde*I and *Xho*I, the DNA fragments and pET21a(+) were ligated with T4 DNA ligase at 16 °C overnight. The ligation product was transformed to 200 µl *E. coli* BL21(DE3) competent cells using a standard procedure. Recombinant plasmid was extracted from positive transformants, and gene accuracy was confirmed by DNA sequencing with an ABI 3730 automated sequencer (Genewiz Inc). Transformant containing pET21a(+)-C2-Xyn was incubated until the cell OD_{600} reached 0.6. Enzyme expression was induced by 1 µM IPTG at 25 °C for 6 h. Cells were collected and lysed ultrasonically. Enzyme was collected and purified using Co^{2+}-binding resin (Amersham Bioscience), because a 6-His tag was added at the

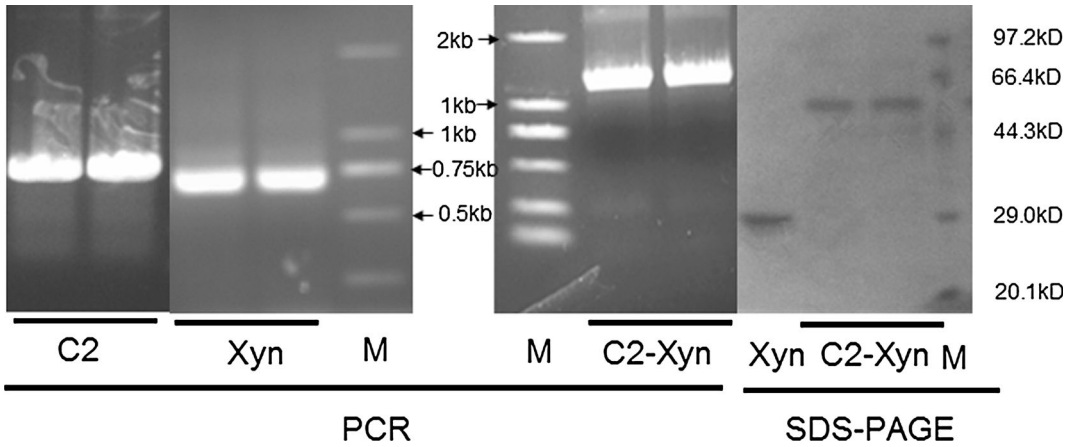

Fig. 1 Construction of the C2-Xyn. The C2-Xyn DNA was created by an overlap extension PCR. The DNA fragments C2 and Xyn had homologous region introduced by the primers P2/P3. The chimeric C2-Xyn enzyme created a band at 55 kDa on a 12 % SDS-PAGE

xylanase *C*-terminus. Active fractions were collected and further purified using Sephadex G-25.

Enzyme properties

Enzyme activities were assayed in parallel with the wild-type Xyn using the DNS method (Liu et al. 2011). Each value was an average of three independent assays. Activities were assayed from pH 2.6 to 4.4 and from 38 to 54 °C on beechwood xylan (Sigma) in phosphate buffer. Enzyme thermostability was determined by assaying residual activities after incubation at 50 °C for 10 min; the $t_{1/2}$ was calculated using the Arrhenius function ($y = A \times \exp^{-x/t}$) (OriginPro 8). Kinetics was assayed under optimal conditions at substrate concentration ranging from 0.625 to 12.5 mg/ml, and the data were fitted to the Hill function: $y = V_{max} \times [S]/(K_m + [S])$ (OriginPro 8). Kinetics were also assayed on oat spelt xylan to analyze enzyme affinity for insoluble substrate. Protein concentration was assayed by the Bradford method. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol xylose after hydrolysis for 1 min under optimal conditions.

Results

Construction of the C2-Xyn

The chimeric C2-Xyn DNA created a band at ~1.2 kb on a 1.4 % agarose gel (Fig. 1), approximately the combined molecular masses of contributing gene fragments C2 (~750 bp) and Xyn (~700 bp). The C2-Xyn DNA was cloned in pET21a(+) and transformed BL21(DE3) *E.coli* competent cell. Recombinant plasmid pET21a(+)-C2-Xyn was extracted from a positive transformant, and gene accuracy was confirmed by DNA sequencing analysis. Having 403 amino acid residues, the chimeric enzyme C2-Xyn created a band at ~55 kDa (Fig. 1), about two times larger than the 185 residue Xyn (~29 kDa).

Enzyme properties

The C2-Xyn had maximal activity at pH 3.6 and 50 °C (Fig. 2; Table 1). The *N*-terminal C2 had caused a 0.2 unit shift in the optimal pH (pH_{opt}) towards more acidic conditions. The *N*-terminal C2 caused an improvement of 2 °C in the optimal temperature (T_{opt}) of the enzyme, similar to the fusion of CBM9 at the *C*-terminus (Liu et al. 2011). It seemed that the *N*-terminal C2 covered partly, and therefore protected, the xylanase at a 2 °C higher reaction temperature.

At 50 °C, the C2-Xyn $t_{1/2}$ was 58.7 min, 2.8 times longer than that of the Xyn (Fig. 2). The *N*-terminal C2 improved both T_{opt} and thermostability of the Xyn. In contrast, the *C*-terminal C2 only improved enzyme thermostability (Liu et al. 2012b). T_{opt} and $t_{1/2}$ are related but different parameters with the former indicating enzyme adaptivity to a higher temperature and the later, enzyme longevity at a certain temperature.

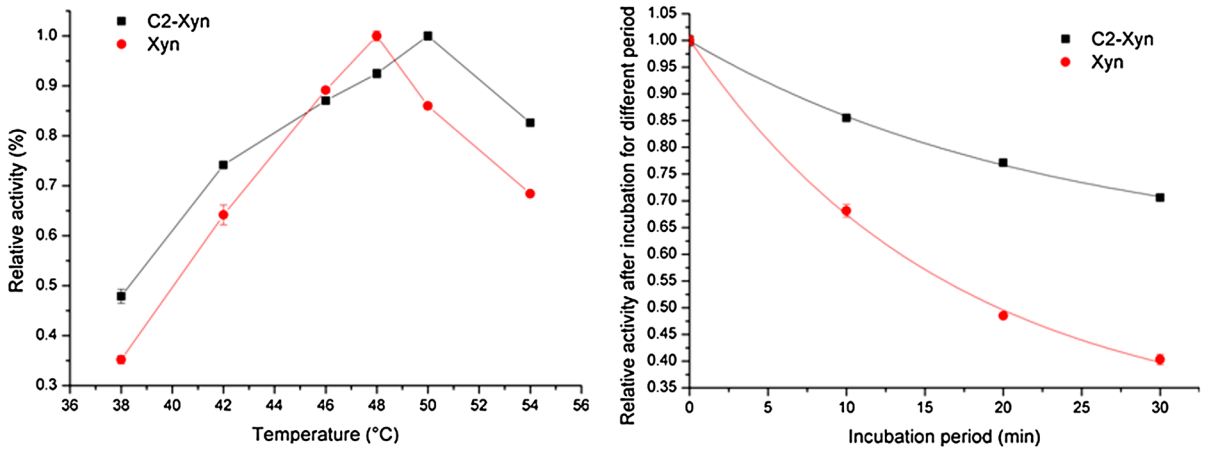

Fig. 2 The C2-Xyn T_{opt} and thermostability. Enzyme activities were assayed at temperatures from 38 to 54 °C (*left*) in phosphate buffer. The C2-Xyn had a 2 °C higher T_{opt} than the Xyn. Residual activities were assayed after incubation at 50 °C for a 10 min interval (*right*). The data were fitted to the equation: $y = A \times \exp(-x/t)$ (OriginPro8). Thermal in-activation half-life ($t_{1/2}$) values were calculated to be 58.7 and 21 min for the C2-Xyn and Xyn, respectively

Table 1 Enzyme properties of the C2-Xyn

	pH_{opt}	T_{opt} (°C)	$t_{1/2}50$ (min)	K_m beechwood/oat spelt xylan	K_{cat} beechwood/oat spelt xylan	V_{max} beechwood/oat spelt xylan
C2-Xyn	3.6	50	58.7	2.9/7	1263/1884	9.3/1.55
Xyn	3.8	48	21	2.7/7.8	282/2113	6/2

pH_{opt} and T_{opt} pH and temperature at which enzyme had maximal activity, $t_{1/2}$ thermal inactivation half-life calculated after incubation at 50 °C, K_m (mg/ml), V_{max} (μmol/min), K_{cat} (s^{-1}) enzyme kinetic parameters calculated after analysis

Enzyme kinetics

Using beechwood xylan as substrate, C2-Xyn exhibited a 155 % maximal catalytic velocity (V_{max}) and a similar K_m value with the Xyn (Fig. 3; Table 1). The similar K_m value indicated that the *N*-terminal C2 contributed a little to enzyme affinity for the soluble substrate. The ~4.5 fold higher K_{cat} value showed that the *N*-terminal C2 greatly improved catalytic efficiency of the xylanase. This effect can be attributed to an enlarged catalytic range and cooperation of the modules C2 and Xyn. The C2-Xyn had a two fold larger size, therefore, a two fold larger catalytic range than the Xyn. In addition, C2 adsorbs with and transfers substrate to the Xyn module. Consistent with the result, all the fusions of CBM9, C2, and C1 at the *C*-terminus improved activity of the GH11 xylanase (Liu et al. 2011, 2012b). Deletion of the CBM32 indicated that it directly participated in substrate recognition and catalysis (Kimiya et al. 2014). Other CBM fusions also improved catalytic activities of the related enzymes (Furtado et al. 2015; Khan et al. 2013; Kittur et al. 2003; Li et al. 2015; Mai-Gisondi et al. 2015; Mangala et al. 2003; Ye et al. 2011).

Using oat spelt xylan as substrate, the C2-Xyn displayed a decreased K_m value and a decreased K_{cat} value (Table 1, Fig. 3). The former and latter value, respectively, indicated that the C2-Xyn had an increased binding affinity and a slightly reduced catalytic efficiency. Perhaps, the increased affinity interfered with enzyme being released from oat spelt xylan. Consistent with the result, a sequential deletion mutants showed that V_{max} of the *T. maritima* xylanase XynA increased in the order of XynAΔC < XynAΔA1C < XynAΔNC (Kleine and Liebl 2006). The *N*-terminal C2 thus improved enzyme affinity for insoluble substrate.

Intending to infer structural changes of the catalytic module of C2-Xyn, its structure was made using a

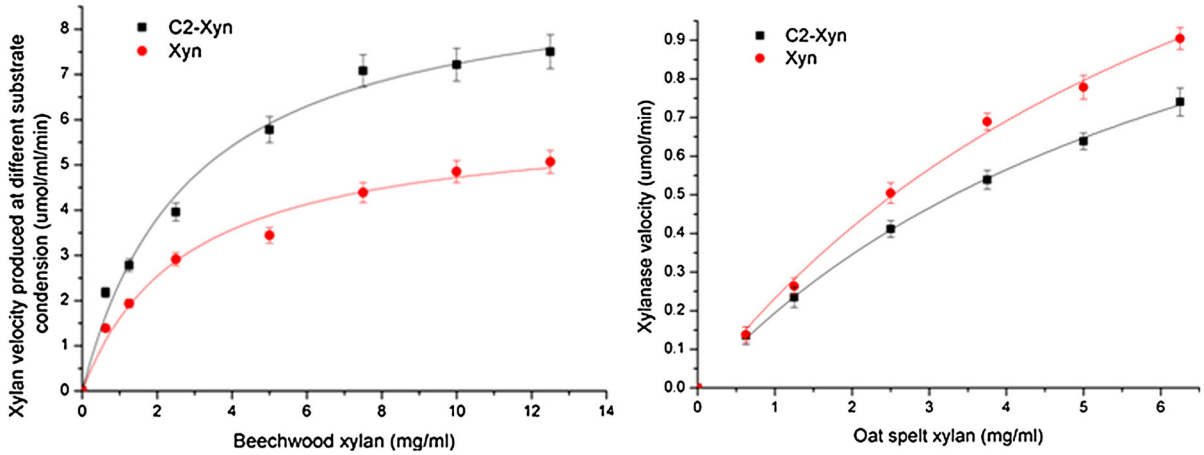

Fig. 3 Kinetics of the C2-Xyn. Enzyme activities were assayed on beechwood (*left*) and oat spelt xylan (*right*) at substrate concentration from 0.625 to 12.5 mg/ml. The data were fitted to the Hill's function: $y = V_{max} \times x/(k_m + x)$ (OriginPro 8). The V_{max} and K_m values on beechwood xylan were 9.33 and 6.02 μmol/min, 2.89 and 2.74 mg/ml for the enzymes C2-Xyn and Xyn, respectively. The V_{max} and K_m values on oat spelt xylan were 1.55 and 2.03 μmol/min, and 6.98 and 7.76 mg/ml for the enzyme C2-Xyn, respectively

homologous modeling procedure. The modeled structure displays a typical β-jelly roll of a family GH11 xylanase (Supplementary Fig 1), and is consistent with the assayed properties of C2-Xyn. Alignment analysis shows that the active sites are Glu297 (proton donor) and Glu388 (nucleophile). The thumb region is composed of ten amino acid residues (Asn335Glu336Pro337Ser338Ile339Thr340Gly341Thr342Ser343Thr344). The *N*-terminal C2 locates near to the substrate-binding residues (Asp255, Trp290, Tyr293, Tyr299, and Gln347), therefore, improves enzyme binding affinity for oat spelt xylan.

Discussion

Both the *N*- and *C*-terminal fusions of C2 improved the Xyn thermostability, indicating that the C2 is a thermostabilizing CBM distinct from those binding only to the substrate (Furtado et al. 2015; Khan et al. 2013; Kittur et al. 2003; Mai-Gisondi et al. 2015; Mangala et al. 2003). In addition, the *N*-terminal C2 improved enzyme T_{opt} by 2 °C. Therefore, the *N*-terminal C2 is a little better for thermal adaptivity of the enzyme than the *C*-terminal C2. A slight difference between the N- and *C*-terminal fusions can be attributed to β jelly-roll structure of the GH11 xylanase and terminal effect of the C2. Probably, a jelly-roll structure folds not as easily as a $(\beta/\alpha)_8$ structure of GH10 xylanase.

It is no surprise that the C2-Xyn exhibited different affinities for beechwood and oat-spelt xylan. The C2 bound specifically to the reducing ends of amorphous cellulose, crystalline cellulose, and unmodified insoluble fraction of oat-spelt xylan, whilst binding weakly to soluble glucans, xyloglucan, and barley α-glucan (Boraston et al. 2001). Within a solvent-exposed slot sufficient enough to accommodate a disaccharide, a CBM interacts with a carbohydrate ligand through an intricate hydrogen-bonding network mainly involving charged residues (Fisher et al. 2015; Nishijima et al. 2015), as well as stacking interactions of Trp175 and Trp71 (Notenboom et al. 2001).

Modular function can be elucidated by truncation and fusion analysis. Truncation mutants have often been made (Kleine and Liebl 2006; Paloheimo et al. 2007; Wang et al. 2014). Removal of some CBMs affects the thermostabilities of the associated xylanases and, thereby, some CBM22 s were originally described as thermostabilizing domains (Charnock et al. 2000). These modules were later recognized as CBMs (Dias et al. 2004; Sunna et al. 2000), and the thermostabilizing effects of CBM deletions were regarded as lacking discrete linker peptides separating from the catalytic domains (Dias et al. 2004; Sunna et al. 2000). Recently, fusion mutants were more often

made (Furtado et al. 2015; Khan et al. 2013; Kittur et al. 2003; Liu et al. 2011, 2012a, b; Mai-Gisondi et al. 2015; Mangala et al. 2003; Tang et al. 2014; Voutilainen et al. 2014; Ye et al. 2011), and fusion seems better for elucidating modular functions. Each module should be separated by a proper linker to provide sufficient space for a module to fold into an intact conformation. Lack of proper linkers might be a reason why transposition of the catalytic modules got unexpected results (An et al. 2005; Hong et al. 2006; Liu et al. 2012a). The natural 22 amino acid linker used in the present study is rich in prolines, glutamines, and valines.

Conclusion

Fusion of the C2 at the *N*-terminus improved the T_{opt} by 2 °C: 2.8-times in thermostability, and 4.5-times in catalytic efficiency of the GH11 xylanase. Thus, the C2 was confirmed to be a thermostabilizing CBM acting at either terminus of the GH11 xylanase. The bifunctional C2 is useful for engineering enzyme thermostability and activity. A modular function might be elucidated better by fusion rather than deletion analysis.

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Supporting information Supplementary Fig. 1—Modeled structure of the catalytic domain of C2-Xyn.

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