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Sandwich fusion of CBM9_2 to enhance xylanase thermostability and activity

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ABSTRACT

Used as model for sandwich fusion, a mesophilic *Aspergillus niger* GH11 xylanase (Xyn) was fused into C2-Xyn-C2 with a thermophilic *Thermotaga maritima* GH10 xylanase carbohydrate-binding module CBM9_2 (C2). Linearized plasmids C2-pET20b-C2-Xyn were amplified from template pET20b-Xyn-C2 with a 4.3 kb C2-pET20b megaprimer, ligated into circular plasmids in blunt-end ligation, and transformed into *E. coli* BL21 (DE3) cells. The C2-Xyn-C2 had optimum activity at 45 °C and pH 4.2, a 2.85 h thermal inactivation half-life at 80 °C and a 8.69 h at 50 °C, with the 8.69 h value 24.8-, 7.5-, and 7.1-fold longer than the Xyn and single terminal fusion enzymes Xyn-C2, and C2-Xyn. Thermodynamics showed that the enzyme had a 1.8 °C higher melting temperature, lower values ΔS, ΔΔG, and a denser structure than the Xyn. Kinetics showed that the C2-Xyn-C2 catalytic efficiency was 1.2–~6-fold and 2.7–~7.9-fold higher on beechwood and oat-spelt xylan than those of the enzymes Xyn, Xyn-C2, and C2-Xyn. The sandwich fusion evolved the xylanase with "armor-hands" to enhance simultaneously thermostability and activity in quality.

1. Introduction

Enzyme is widely used in biotechnology, and wide application demands for thermostable enzymes. Enzyme thermostability has been rationally engineered, such as disulphide bond introduction [1–6], computational design [7–10], site-directed mutation [11,12], and disordered terminal residue deletion [13,14]. Relatively, a more straightforward strategy is fusing a thermophilic domain. Carbohydrate-binding module (CBM) is a non-catalytic domain usually attached to a hydrolase. A CBM9 locates natively at the C-terminus of a *Thermotaga maritima* thermophilic GH10 xylanase, and contains a sub-module CBM9_2 (C2) [15,16]. When the C2 was fused at the C– or N-terminus of an *Aspergillus niger* xylanase (Xyn), a typical GH11 family xylanase that usually has no CBM, the Xyn thermostability was enhanced [17–19].

However, all the CBMs are fused at an enzyme one terminus and are usually consistent with their native locations relative to catalytic domains, and these one terminal fusions only moderately enhanced enzyme thermostabilities or activities [17–22]. A novel concept is sandwich fusion, i.e., fusing a CBM at an enzyme both termini. To check the concept and effect on enzyme property, an ideal model is fusing the mesophilic Xyn with the similar size thermophilic C2 into a C2-Xyn-C2 enzyme through a natural linker-peptide from the *T. maritima* GH10 xylanase. The 193 residue Xyn has a 17.6 min thermal inactivation half-life ($t_{1/2}$) at 50 °C, and the 195 residue C2 has a denaturation temperature above 100 °C [15–19]. The sandwich fusion simultaneously enhances the Xyn thermostability and activity in quality instead of only in quantity.

2. Materials and methods

Q5 DNA polymerase, dNTPs, T4 DNA ligase, and *Dpn*I were purchased from NEB (Beijing, China). pET20b-C2 and pET20b-C2-Xyn were self constructed. Primers PC2P (p5′-CATATGATGGTAGCGACAGC-3′, forward)/VR-P (p5′-GTATATCTCCTTCTTAAAGTTAAACAAAATTATT TCTAG-3′, reverse)/RX-P (p5′-AGAGGAGATCGTGACACTG-3′, forward) were synthesized and phosphorated for blunt-ended ligation (Genewiz, China).

2.1. Recombinant plasmid construction

2.1.1. Megaprimer amplification

The pET20b-C2-Xyn-C2 plasmid was constructed by a two-step PCR [24]. A 4.2 kb linearized C2-pET20b DNA was amplified from the template pET20b-C2 in a 1st-PCR with primers PC2P and VR-P to serve as 2nd-PCR megaprimer. Reaction was carried out in a 50 μL volume containing 40 ng pET20b-C2, 500 nM primers PC2P and VR-P, 1 U Q5 DNA

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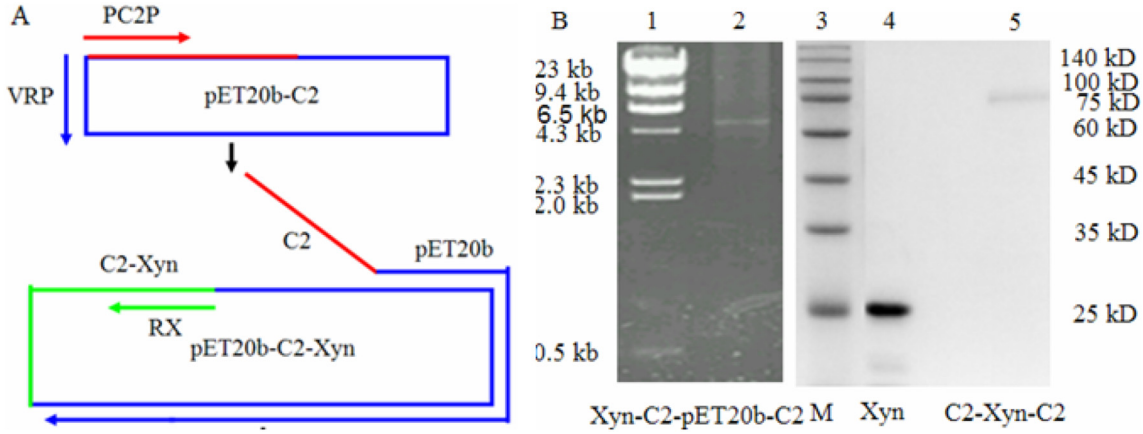

Fig. 1. Construction of the C2-Xyn-C2. (A) Plasmid construction. A 4.3 kb C2-pET20b DNA was amplified in the 1st-step with the primers PC2P and VRP, and served as megaprimer in the 2nd-step to amplify linearized C2-pET20b-C2-Xyn plasmids from the template pET20b-C2-Xyn by coupling with the primer RX. (B) PCR amplification and SDS-PAGE, DNA (BL1) and protein marker (BL3). The amplified C2-pET20b-C2-Xyn linearized plasmids created a 5.5 kb band (BL2). The C2-Xyn-C2 created a 75 kDa band on a 12% SDS-PAGE gel (BL5), larger than the Xyn (BL4).

polymerase, 200 μM dNTPs, and 1 × polymerase buffer. PCR procedure was: pre-denaturation at 98 °C for 3 min, 30 cycles of denaturation at 98 °C for 30 s, annealing at 64 °C for 20 s, and extension at 72 °C for 149 s, extension at 72 °C for 10 min, and stored at 4 °C. The C2-pET20b DNA was purified from gel-electrophoresis with DNA clean kit [Qiagen, China].

2.1.2. Plasmid amplification

Linearized plasmids C2-pET20b-C2-Xyn were amplified from the template pET20b-C2-Xyn in a 2nd-PCR with the C2-pET20b megaprimer and a reverse primer RX-P. Reaction was carried out in a 50 μL volume containing 80 ng pET20b-C2-Xyn templates, 240 ng C2-pET20b forward megaprimer, 500 nM primer RX-P, 1 U Q5 DNA polymerase, 200 μM dNTPs, and 1 × buffer. PCR procedure was: pre-denaturation at 94 °C for 5 min, 15 cycles of denaturation at 94 °C for 30 s, annealing at 80 °C for 30 s, RAMP with 0.2 °C/s, and annealing at 64 °C for 20 s, extension at 72 °C for 190 s, extension at 72 °C for 10 min, and stored at 4 °C.

2.1.3. Blunt-ended ligation

Ligation was carried out at 16 °C for 16 h in a 10 μL volume containing 60 ng linearized C2-pET20b-C2-Xyn purified with DNA clean kit (Axygen, US), 700 U T4 DNA ligase, and 1 × buffer. Thereafter, 2 U *Dpn*I was added to digest template plasmids at 37 °C for 3 h, and the product was transformed into 200 μL *E. coli* BL21(DE3) home-made competent cells in a standard 42 °C heat-shock method. After positive transformant selection, recombinant plasmid was extracted and Sanger sequenced (Genewiz, China).

2.2. Protein expression and purification

The accurate transformant was cultivated in LB medium at 37 °C and 220 r/min. Once the culture OD_{600} reached to 0.6, 0.5 mM IPTG was added to induce protein expression at 25 °C for 5 h. Thereafter, cells were collected and lysed by sonication. His-tagged proteins were bound to Ni^{2+}-binding resin (Amersham Biosciences), washed processively with 10-fold volumes of 20, 30, and 50 mM imidazole elution buffers, and eluted with 100 mM imidazole buffers. After excluding imidazoles, the purified enzymes were detected with 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. Protein concentration was measured by the Bradford method.

2.3. Enzyme property assay

Enzyme property was assayed by the 3, 5-dinitrosalicylic acid (DNS) method against beechwood xylan [13,18]. All experiments were conducted in triplicate, and an average was reported for each value. The optimum pH (pH_{opt}) was determined at 50 °C in different pH phosphate buffers ranging from 3.0 to 4.8. The optimum temperature (T_{opt}) was determined in pH 4.2 buffers at temperature ranging from 38 °C to 51 °C.

Thermostability was determined by assaying respectively residual activities after incubation at 50 °C and 80 °C for different periods, and mineral oil was added to exclude interference of water evaporation. Thermal inactivation half-life ($t_{1/2}$) was calculated by fitting the data with the thermal decay equation $y = A * \exp(-x/t)$.

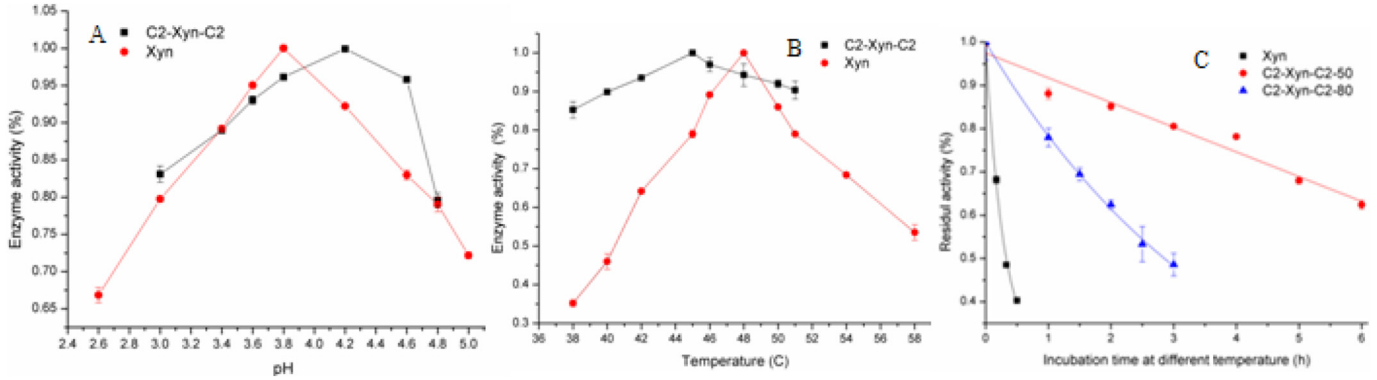

Fig. 2. Enzyme properties. Enzyme activity was assayed against beechwood xylan at different pH phosphate buffers (A) and different temperatures (B). Thermostability (C) was determined at 50 °C (C2-Xyn-C2_50) and 80 °C (C2-Xyn-C2_80).