Process Biochemistry 46 (2011) 395–398

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Directed evolution of a mesophilic fungal xylanase by fusion of a thermophilic bacterial carbohydrate-binding module

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ARTICLE INFO

Article history:
Received 13 March 2010
Received in revised form 26 June 2010
Accepted 23 July 2010

Keywords:
GH11 xylanase
CBM9
Domain fusion
Thermo-activity
Catalytic efficiency

ABSTRACT

Bacterial GH10 xylanase usually contains carbohydrate-binding module (CBM) that binds to insoluble xylan. Differing from GH10 xylanase, we isolated a fungal GH11 xylanase containing a single catalytic domain from *Aspergilus niger* (XYN). The thermophilic CBM from *Thermotoga maritima* (TmCBM9-1_2) might increase the mesophilic XYN's thermo-activity and catalytic efficiency on insoluble xylan, we fused it with the TmCBM9-1_2 behaving as "hand" to grasp xylan actively. The chimeric xylanase XYN-TmCBM9-1_2 exhibited an optimal activity at pH4.2 and 49 °C, 2 °C higher than the thermo-activity of XYN. The chimeric xylanase's activity was 970.1 ± 5.8 U/mg on insoluble oat-spelt xylan, 4.2-fold of that on soluble birchwood xylan (228.1 ± 1.1 U/mg). In contrast, the XYN's activity was 226.9 ± 1.2 U/mg on insoluble oat-spelt xylan, only 40% of that on soluble birchwood xylan (567.2 ± 3.0 U/mg). Fusing with the TmCBM9-1_2 increased the XYN's property, indicating that we can direct to evolve a molecule's function through fusing domains of bacteria and fungi.

1. Introduction

Carbohydrates are energy resource in nature. However, carbohydrates are complicate and hard to break down. Thus, carbohydrate hydrolases are important in energy recycling. Xylanase (EC 3.2.1.8) is an important carbohydrate hydrolase that breaks down xylan, a major constituent of hemicelluloses. Thereby, xylanase is widely used in feed, flour, baking industry, pulp bleaching, and renewable energy. However, industrial environments are often tough, such as pulp is made at a high temperature (95–100 °C); while xylanase generally has an optimal temperature (T_{opt}) between 50 °C and 60 °C, unfair for using at a higher temperature. Thermophilic proteins had many features adapting to a higher temperature, such as a large number of charged residues, additional hydrogen bonds, increase of intra-molecular hydrophobic packing, and so on [1–4]. Bioinformatics was also used to find characteristic dipeptides correlating with amylase and xylanase function [5–7]. Yet, to improve xylanase's thermo-activity is still difficult because of many influence factors. Moreover, xylan often binds with cellulose, making it hard to break down by xylanase.

To hydrolyze insoluble cellulose, cellulase usually contains non-catalytic domains known as cellulose-binding domains (CBD), since it showed affinity against cellulose. Thereafter, the discovery of new domains binding carbohydrates other than cellulose, led to a new classification as carbohydrate-binding module (CBM), which was important for hydrolyzing insoluble substrate [8]. There were 55 defined families of CBMs displaying variation in ligand specificity (CAZy database, 2009) [9]. CBM was useful for increasing enzyme's binding efficiency to crystal micro-cellulose [10]. Because GH10 xylanases from bacteria usually contain multiply domains including CBM, its function was investigated by adding or removing from bacterial multi-domain GH10 xylanases [11–14]. For example, the fusion of CBM-VI increased the *Bacillus halodurans* xylanase's binding efficiency to xylan [11]. Thereafter, the removal of CBM22 decreased the *Clostridium thermocellum* xylanase's T_{opt} from 75 °C to 65 °C [12], indicating some CBMs might connect with enzyme's thermo-activity. Thus, CBM might be used to evolve a xylanase's thermo-activity or catalytic efficiency on insoluble xylan.

Different from bacterial GH10 xylanase's multiple domains, we isolated a single-domain fungal GH11 xylanase from *Aspergillus niger* (XYN) having high catalytic efficiency on soluble birchwood xylan, which was a mesophilic enzyme having 185 amino acids [15]. With the idea that a thermophilic CBM might evolve the XYN's thermo-activity and catalytic efficiency on insoluble xylan, we isolated the CBM9 containing two tandem ~170 amino-acid binding modules (TmCBM9-1_2) from a thermostable GH10 xylanase having the T_{opt} at ~90 °C, which was isolated from hypo-thermophilic bacteria, *Thermotoga maritima* [16]. As the TmCBM9-1_2 was ther-

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doi:10.1016/j.procbio.2010.07.026

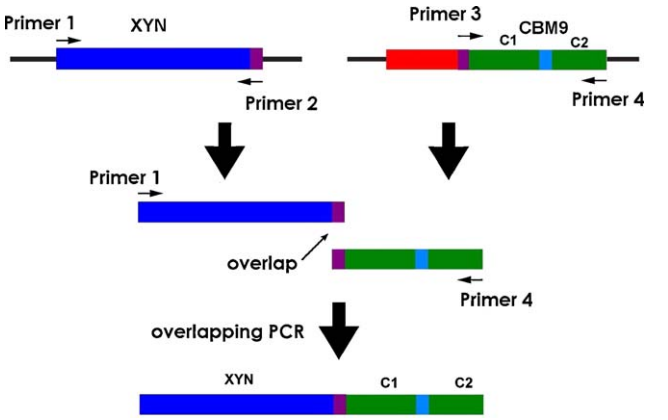

Fig. 1. Fusion of gene *xyn* with *TmCBM9-1_2*, the two genes were fused together by overlapping-extension PCR in two steps using primer 1, 2, 3 and 4.

mophilic and had cellulose-binding properties [17], we fused it to the XYN behaving as "hand" to grasp xylan actively. The study was useful for chimeric protein expression, as producing soluble and active chimeric carbohydrate proteins were difficult [8].

2. Material and method

2.1. Bacterial strains and reagents

We deposited the isolated XYN gene (*xyn*) from *Aspergillus niger* in GenBank (GenBank accession no. EU375728), which coded for 185 amino acid xylanase. According to protein annotation and gene sequence of the *T. maritima* xylanase (Swiss-prot accession no. Q60037, GenBank accession no. Z46264), we isolated the thermophilic TmCBM9-1_2 gene (*cbm9-1_2*), which contained two tandem repeats of family 9 CBM (formerly known as C1 and C2) preceded by a 29 amino-acid linker sequence. T4 DNA ligase, DNA marker, protein marker and other reagents were used in gene recombination (Takara Inc., Dalian, China).

2.2. Construction and purification of the chimeric xylanase XYN-TmCBM9-1_2

We fused *xyn* with *cbm9-1_2* by overlapping-extension PCR in two steps (Fig. 1). Firstly, the *xyn* and the *cbm9-1_2* gene were amplified separately using 3.0 μl pET20b-*xyn* or pET20b-*cbm9-1_2* as template and p1/p2 or p3/p4 as primers (each 1.0 μl) with polymerase mixtures (0.5 μl Pfu polymerase, 4.0 μl dNTPs and buffer), through 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 41 °C), and extension (1 min at 72 °C). After gel-separation, the two genes were recovered using DNA purification kit.

Secondly, the recovered *xyn* and *cbm9-1_2* gene served as template (each 2.0 μl) and p1/p4 as primers (each 1.0 μl) with polymerase mixtures (0.5 μl Pfu polymerase, 4.0 μl dNTPs and buffer). The chimeric gene was amplified through 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 52 °C), and extension (1.5 min at 72 °C) (Fig. 1). The primers were shown in italic for *Nde*I and *Xho*I restriction sites, and in bold for homologous region:

p1: 5′ -att*ccatatg* agtgccggtatcaac-3′
p2: 5′ -ggg**tggaaggacctcagg agaggagatcgtgacac**-3′
p3: 5′ -cca**gtgtcacgatctcctct cctgaggtccttcca**-3′
p4: 5′ -atta*ctcgag* cttgatgagcctgag-3′

Thereafter, the chimeric gene *xyn-cbm9-1_2* was cloned into pMD18-T vector, and transformed *E. coli* JM109 competent cells. The recombinant plasmid pMD18-T-*xyn-cbm9-1_2* was isolated and digested with *Nde*I and *Xho*I. After that, the chimeric gene was cloned into pET20b (Novagen, Shanghai, China), which was digested with *Nde*I and *Xho*I to delete the redundant restriction endo-nuclease sites (Supplementary Fig. 1). After transforming *E. coli* BL21(DE3) competent cells, the recombinant plasmid pET20b-*xyn-Tmcbm9-1_2* was sequenced to confirm gene accuracy by using an ABI 3730 automated sequencer (Invitrogen Biotechnology, Shanghai, China). The accurate transformant was grown in LB at 37 °C till cell culture to OD_{600} = 1.0, and xylanase was induced by adding isopropyl-beta-D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM. After further incubation for 5 h at 30 °C, cells were harvested and lysed by sonication. After removing cell debris, enzyme-containing supernatant was applied to column containing Co^{2+}-binding resin. As polyhistidine tag (6 × His) was added to the C-terminal of XYN-TmCBM9-1_2 and XYN, both xylanases were purified by affinity chromatography method. Afterwards, the purified xylanase was detected using 12% polyacrylamide SDS-PAGE, and xylanase's binding to xylan was determined by affinity electrophoresis using 10% native polyacrylamide gel containing 0.1% oat-spelt xylan.

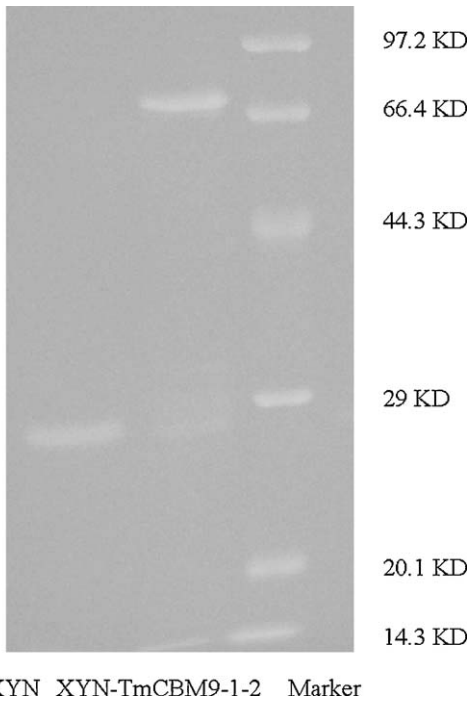

Fig. 2. SDS-PAGE of the XYN-TmCBM9-1_2, the chimeric xylanase had a molecular mass about 3-folds that of the XYN, both xylanases had apparent molecular masses bigger than their theoretical molecular masses.

2.3. Assay of the chimeric xylanase property

The chimeric XYN-TmCBM9-1_2 was assayed in parallel with XYN for properties, such as T_{opt} to indicate thermo-activity, residual activity to describe thermostability, which was assayed by incubating for different length of time (0–30 min) at 50 °C and expressed as ratio in percentage of the activity of untreated xylanase. pH profile was assayed in 50 mM imidazole-biphthalate buffers at each pH value (pH 2.6–7.0). Protein concentration was measured by Spectrophotometer ND-1000 at 280 nm using derivatization (NanoDrop Technologies, Wilmington, United States). Xylanase specific activity was assayed on insoluble oat-spelt xylan and soluble birchwood xylan (Sigma-Aldrich, Shanghai, China). Xylanase standard activity was determined by dinitrosalicylic acid method (DNS). Mixture of 100 μl enzyme and 100 μl 2% birchwood xylan was reacted for 16 min at related temperature and the reaction was stopped by adding 600 μl DNS followed by boiling for 15 min. The color developed, representing xylanase activity, was measured by spectrophotometer at 550 nm. One unit of xylanase activity (U) was defined as the amount of enzyme that produced 1 μmol of xylose per minute.

3. Result

3.1. The fusion gene and the chimeric xylanase

After gel-electrophoresis of PCR products, the fusion gene *xyn-cbm9-1_2* produced a specific DNA band about 1.7 kb, equaling to nucleotide addition of these two genes (Supplementary Fig. 2). Thereafter, positive transformant was selected and grown to extract recombinant plasmid pET20b-*xyn-cbm9-1_2*, which was sequenced to confirm the gene accuracy [GenBank accession no. GQ169711]. The chimeric XYN-TmCBM9-1_2 produced a specific protein band about 72 kD after SDS-PAGE, 3-fold that of XYN (28 kD) (Fig. 2, Table 1), because the TmCBM9-1_2 had 2-fold amino acids more than XYN. Both xylanases showed apparent molecular masses bigger than their theoretical masses (62.7 kD for XYN-TmCBM9-1_2 and 20.8 kD for XYN). Previous study found acidic proteins having higher apparent molecular masses on SDS-PAGE [18,19]. As both the wild and chimeric xylanases were acidic (pI-XYN = 4.47, pI-XYN-TmCBM9-1_2 = 4.67), their higher apparent molecular masses