Journal of Molecular Catalysis B: Enzymatic 113 (2015) 29–38

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Metagenomics of un-culturable bacteria in cow rumen: Construction of *cel*9E–*xyn*10A fusion gene by site-directed mutagenesis

Young Min Kang[a], Min Keun Kim[b], Jin Mi An[c], Md. Azizul Haque[d], Kye Man Cho[d,*]

[a] Herbal Medicine Resources Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine (KIOM), Daejeon 305-811, Republic of Korea
[b] Environment-Friendly Research Division, Gyeongsangnam-do Agricultural Research and Extension Service, Jinju 660-360, Republic of Korea
[c] Research Institute of Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea
[d] Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 660-758, Gyeongnam, Republic of Korea

ARTICLE INFO

Article history:
Received 31 August 2014
Received in revised form
16 November 2014
Accepted 25 November 2014
Available online 4 December 2014

Keywords:
*cel*9E–*xyn*10A
Cow rumen
Fusion gene
Metagenomics
Unculturable bacteria

ABSTRACT

The metagenomes of complex microbial communities are rich sources of novel biocatalysts. Genetic engineering facilitates the artificial fusion of genes that encode functional proteins into a single open reading frame. The cloning of the cellulase and xylanase genes from the cow rumen metagenome resulted in the construction of a bifunctional fusion gene via site-directed mutagenesis for further specific industrial processes. The metagenome of cow rumen bacteria was the source of a gene that encodes an extracellular β-glucanase for cloning and expression in *Escherichia coli* DH5α. The cellulase (*cel*9E) gene of un-culturable rumen bacteria existed in tandem with the xylanase (*xyn*10A) gene. The genes were 2268 bp and 1.578 bp and encoded 756- and 526-aa proteins, respectively. BLAST analyses and domain predictions assigned Cel9E and Xyn10A to glycosyl hydrolase families 9 and 10. The molecular weight of the individual proteins Cel9E and Xyn10A were estimated to be approximately 76.0 kDa and 56.0 kDa by CMC–SDS-PAGE and OSX–SDS-PAGE, respectively. The 3909 bp *cel*9E–*xyn*10A fusion gene encoded a 1303-amino acid residue protein with a molecular weight of approximately 137.0 kDa according to CMC/OSX–SDS-PAGE. The maximum cellulase and xylanase activities from the fusion protein Cel9E–Xyn10A were observed at pH 6.0 and pH 8.0, respectively. The optimal temperature for the bifunctional enzyme was found to be 50 °C. The improved catalytic efficiency of the Cel9E–Xyn10A for the cellulase and xylanase activity was equivalent to 1.47- and 2.21-fold of the parental efficiency. We report the presence of the *cel*9E gene in tandem with the *xyn*10A gene in the metagenome of un-culturable cow rumen bacteria. The construction, expression and characterization of the *cel*9E–*xyn*10A bifunctional gene fusion obtained by site-directed mutagenesis are also reported.

1. Introduction

During the past decade, the molecular diversity of ruminal microorganisms has been extensively explored by adopting cultivation-independent analyses of microbial rRNA sequences to screen for useful genes from uncultivated microbial groups in order to represent the majority of indigenous microbial communities. However, the analysis of the rumen metagenome is complicated by the fact that most microorganisms (85–95%) cannot be cultured [1,2] and these microorganisms probably comprise more than 1000 individual species of bacteria, fungi, and protozoa [3]. The rumen microbial population presents a rich and, until recently, underutilized source of novel enzymes with tremendous potential for industrial application.

The enzyme activities confirmed to exist in the rumen are diverse and include plant cell wall polymer-degrading enzymes (e.g., cellulase, xylanase, β-glucanase, pectinases), amylase, protease, phytases, and tannases. The variety of enzymes present in the rumen arises not only from the diversity of the microbial community but also from the multiplicity of fibrinolytic enzymes produced by individual microorganisms [4]. Recently, novel plant cell-wall degrading enzymes and genes have been discovered in un-culturable microorganisms from the rumen metagenome [5]. Enzymes that catalyze the hydrolysis of plant cell-wall polysaccharides, which enable the conversion of biomass for the production of fuels and other chemicals, are of growing industrial interest [6]. Cellulose is an un-branched glucose polymer composed of anhydro-β-1,4-glucose units linked by a β-1,4-glycosidic bond, and xylan has a backbone of β-1,4-linked xylopyranosyl residues. Cellulases

* Corresponding author. Tel.: +82 55 751 3272; fax: +82 55 751 3279.
E-mail address: kmcho@gntech.ac.kr (K.M. Cho).

http://dx.doi.org/10.1016/j.molcatb.2014.11.010
1381-1177/

are found in glycosyl hydrolase families 5, 6, 7, 8, 9, 12, 44, 45, 48, and 61. Some cellulases contain a multi-domain structure, including a catalytic domain (CD), one or more cellulose-binding domains (CBD) and cellulose-binding modules (CBM), cell interaction motifs, linker or repeat regions, and central type III fibronectin (Fn3) modules [7]. Furthermore, xylanases, which degrade the hemicellulose linkers between the lignin and cellulose constituents of plant cell walls, are classified in glycosyl hydrolase families 8, 10, 11, and 43. Moreover, many xylanases are modular enzymes that contain one or more discrete domain(s), such as a carbohydrate-binding module (CBM) in addition to a catalytic domain [8].

Genetic engineering has facilitated the artificial fusion of two genes that encode functional proteins into a single open reading frame. Theoretically, the fusion of two enzymes that catalyze sequential reactions may generate a catalytically bifunctional protein. Furthermore, the catalytic efficiency of the chimeric enzyme might be superior to that of a simple mixture of the individual enzymes [9,34]. Although the natural diversity of enzymes provided some candidates that have evolved multifunctional activity [9,10], most fusion enzymes have resulted from the in vitro fusion of individual enzymes [11,12]. Previously, a number of studies have also shown that fusion proteins could be created by the end-to-end fusion via overlap PCR [13–16]. In this study, the bifunctional fusion gene construction, cloning and sequencing of the cellulase and xylanase genes from the metagemome of un-culturable cow rumen bacteria in a metagenomic library were achieved via site-directed mutagenesis. In addition, the biochemical properties of this *cel*9E–*xyn*10A fusion gene product that is expressed in *Escherichia coli* DH5α have been examined.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and sampling of rumen metagenome

Rumen bacteria (Table 1) were cultured in media recommended by ATCC and DSMZ. *E. coli* DH5α, *E. coli* EPI300™, and recombinant *E. coli* cells were cultured in LB medium (Difco, NJ, USA) containing appropriate antibiotics (ampicillin, 50 μg mL^{-1}; chloramphenicol, 12.5 μg mL^{-1}) at 37 °C. Samples of rumen contents were obtained from a closed herd at the Gyeongnam National University of Science and Technology (Jinju, Korea). Korean native cows (*Bos taurus coreanea*) (body weight approximately 400.0 ± 10 kg) were fed a mixed ration (rice hull and concentrate 4:1 ratio) twice a day. The concentrate was purchased from Daehan Food (Ulsan, Korea). Representative samples of the total rumen contents were collected from the cow via a ruminal fistula prior to the morning feed. The drawn samples were placed on ice, immediately transferred into an anaerobic box and stored at −80 °C.

2.2. Recombinant DNA techniques

Standard protocols were followed for the restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other related techniques [17]. The restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Boehringer Mannheim (Indianapolis, IN, USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Construction of cow rumen metagenomic library

A genomic library was constructed in the cosmid vector pCC1FOS™ as previously described [9,11]. The total genomic DNA from the cow rumen environment was sheared into approximately 23–40 kb fragments using a syringe. The sheared DNA (100 μg) was layered directly onto a 12 mL, 5–40% sucrose gradient in 20 mM Tris–HCl, pH 8.0 and 10 mM EDTA/50 mM NaCl. The samples were centrifuged for 22 h at 22 °C at 25,000 rpm using a SWi-41 rotor (Beckman, CA, USA) and fractionated according to sizes. The fractioned DNA was recovered by precipitation with two equal volumes of ethanol, and the sizes of DNA fragments in each fraction were estimated on a 0.7% agarose gel and then end-repaired to yield blunt 5′-phosphorylated ends. The resulting DNA fragments were ligated and packaged using a lambda DNA packing kit (Epicentre, WI, USA). The packaged sample was transformed into *E. coli* EP1300™ by injection. The library clones were plated on the indicator medium for cellulase and xylanase activities.

2.4. Cloning and sequencing of cel and xyn gene

Sub-cloning in pCYF100 was undertaken by partially digesting it with *Sau*3AI (Promega, USA). Two to five kb fragments of the cosmid DNA from this partial digestion were ligated into the *Bam*HI (Promega, WI, USA) site of pBluescript II SK+ (Stratagene, WI, USA) vector treated with CIP (Promega, WI, USA) and then transformed into *E. coli* DH5α. Positive clones were detected via the growth of bacteria on a cellulase and xylanase indicator medium [LB agar plates containing appropriate antibiotics and 0.5% carboxylmethylcellulose (CMC) and 0.5% oat spelt xylan (OSX)]. The active band appeared as a yellow halo on a red background when bacteria were cultured at 37 °C for 24 h and stained with 0.5% Congo red solution for 30 min [18]. Nucleotide sequences were determined with the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CT, USA). The assembly of the nucleotide sequences and the amino acid sequence analysis were made possible with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). The DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information (NCBI) with the BLAST network service and the non-redundant DNA and protein sequence databases. The nucleotide sequence data reported are available in the GenBank database under the accession number KM12345 for *cel*9E and KM23456 for *xyn*10A.

2.5. Construction of fusion gene

The tandem plasmid, pCY120, encoding cellulase and xylanase from un-culturable rumen bacteria were used for the in vitro site-directed mutation experiments. Site-directed in vitro mutations were conducted employing pCSAY130 with 28-mer synthetic oligonucleotide primers, 5′-ATCGCGGGACAGGCACGGTACATTGGT-3′ (forward, containing a point mutated site as underlined) and 5′-ACCAATGTACCGTGCCTGTCCCGCGATC-3′ (reverse, containing a point mutated site as underlined). The reaction mixtures (50 μL) contained pCSAY120 plasmid (1 μL) DNA (50 ng μL^{-1}), 10 ρmol of each primer (4 μL), 2 mM dNTP mixture (5 μL), 10× *Pfu* DNA polymerase buffer (5 μL) containing 20 mM $MgSO_4$, and 2.5 U of cloned *Pfu* DNA polymerase. The PCR products were incubated on ice for 5 min, treated with 1 μL of *Dpn*I restriction enzyme (10 U/μL) and incubated for 1 h at 37 °C. The resultant *Dpn*I plasmids were transformed into *E. coli* DH5α according to the manufacturer's specifications (site-directed mutagenesis Kit, Stratagene).

2.6. Expression and purification of enzyme

The PCR product generated with the primers, 5′-AAAA-GTCGACCGCGGAAGCGGAACAGGA-3′ (sense, containing a *Sal*I site as underlined) and 5′-AAAACTCGAGCTGTCCCGCGATCAGCAG-3′ (antisense, containing a *Xho*I site as underlined) and 5′-AAAA-GGATCCGCGGGCGAGGCGAAAACG-3′ (sense, containing a *Bam*HI