Synthesizing a Cellulase like Chimeric Protein by Recombinant Molecular Biology Techniques

Hirenda Nath Banerjee*, Christopher Krauss, Valerie Smith, Kelly Mahaffey, and Ava Boston
Department of Natural Pharmacy and Health Sciences, Elizabeth City State University, University of North Carolina, Elizabeth City, NC-27909, USA

Abstract

In order to meet the Renewable Fuels Standard demands for 30 billion gallons of biofuels by the end of 2020, new technologies for generation of cellulosic ethanol must be exploited. Breaking down cellulose by cellulase enzyme is very important for this purpose but this is not thermostable and degrades at higher temperatures in bioreactors. Towards creation of a more ecologically friendly method of rendering bioethanol from cellulosic waste, we attempted to produce recombinant higher temperature resistant cellulases for use in bioreactors. The project involved molecular cloning of genes for cellulose-degrading enzymes based on bacterial source, expressing the recombinant proteins in *E. coli* and optimizing enzymatic activity. We were able to generate *in vitro* bacterial expression systems to produce recombinant His-tag purified protein which showed cellulase like activity.

Introduction

Cheap, clean, green energy production is a goal of Department of energy and EPA. Biofuels are made by converting renewable materials--for example, corn kernels, wood chips left over from pulp and paper production, prairie grasses, and even garbage--into fuels and chemicals. Most biofuels used today are made from the fermentation of starch from corn kernels. That process, although simple, is costly because of the high price of the corn kernels themselves. Agricultural waste, such as corn stover (the leaves, stalks, and stripped cobs of corn plants, left over after harvest), is cheap. These materials are largely composed of cellulose, the chief component of plant-cell walls. Cellulose is far tougher to break down than starch. An additional complication is that while the fermentation reaction that breaks down corn starch needs just one enzyme, the degradation of cellulose requires a whole suite of enzymes, or cellulases, working in concert.
The cellulases currently used industrially, all of which were isolated from various species of plant-decaying filamentous fungi, are both slow and unstable, and, as a result, the process remains prohibitively expensive. Even a two-fold reduction in their cost could make a big difference to the economics of renewable fuels and chemicals; Thermostability is a requirement of efficient cellulases, because at higher temperatures, 70 or even 80 degrees Celsius--chemical reactions are more rapid. In addition, cellulose swells at higher temperatures, which makes it easier to break down. Unfortunately, the known cellulases from nature typically won't function at temperatures higher than about 50°C. Cellulolytic anaerobic bacteria use macromolecular structures known as cellulosomes to hydrolyze recalcitrant cellulosic substrates [1,2]. Within the cellulosome, cellulases and other glycoside hydrolases [3,4] are assembled onto multidomain scaffoldin proteins for efficient degradation of cellulosic substrates [4]. Cellulosome assembly is achieved by binding dockerin domains from enzymes with cohesin domains in scaffoldin, while localization with substrate is mediated by one or more Carbohydrate Binding Modules (CBMs) on the scaffoldin [1,2,5]. The modularity of cellulosomes has spurred interest in ‘designer cellulosomes’ [6], where different cellulases are synthetically combined for a specific application. Within a given glycoside hydrolase family, a diverse pool of potential cellulases would be beneficial for designer cellulosomes by providing a suite of enzymes with differing properties and an extensive platform for further enzyme engineering. Family 48 cellulases (Cel48) are ideal candidates for designer cellulosomes [3]. As one of the most important families of bacterial cellulases, they are usually a major constituent of bacterial cellulosomes [4,7–12]. Of the 116 bacterial Cel48 genes currently predicted in the CAZy database (http://www.cazy.org/) only 13 have been characterized. We chose SCHEMA recombination to plan to synthesize a diverse set of new family 48 sequences. SCHEMA is a structure-guided, site-directed protein recombination method that has been used to generate thousands of novel P450s, β-lactamases, and fungal cellulases. The chimeric proteins that are made by recombining natural sequences differ. Our objective for this project was to construct chimeric synthetic cellulase genes for production of thermostable cellulases for efficient breakdown of cellulose at high temperature.

Materials and Methods

Genomic DNA from bacteria Cellulomonas sp. (ATCC® 21399) was used as a template to do PCR using standard PCR reagents and assay conditions using the primers:

| Primer Name | Sequence 1 | Sequence 2 |
|-------------|------------|------------|
| CCELcdCTHEdock+XbaIfwd | GCAATCTCTTTCCAGATTCTGAAATGACAT | ATAAAGTACCTGGTAGACTTCCTCTACT |
| CCELcdCTHEdock+XbaIrev | AGGTACTTTATATGTCATTCTAGAATCTGGG | AAGAGTATTGCATAAACTCCATTTGC |

The amplicon was further sequenced and the obtained sequence (Figure 1) was subjected to NCBI-BLAST search and showed homology to A. thermophilum celA gene (Figure 2).

The amplicon was then cloned into a Gateway System (Invitrogen, USA) his-tag expression vector and BL-21 E. coli bacteria was transformed with this construct. The bacteria was then
grown in LB medium and IPTG was used to induce the protein, which was then his-tag purified using a nickel column (please see the gel picture in Figure 3), protein concentration was measured by using standard Bradford method (Sigma, USA).

**Cellulase Assay**

**Method**

A standard assay for cellulase activity was performed with a reaction mixture containing 0.52% carboxymethyl cellulose in 10 mM sodium phosphate (pH 7.0) at 30°C. Reduced sugar produced by the reaction was determined using the method described by Park and Johnson [13] using a standard BioRad (USA) spectrophotometer.

**Results and Discussion**

We were interested to synthesize a chimeric synthetic cellulase gene from the different cellulases DNA sequence that are there in the gene bank to produce a thermostable cellulose, our initial bioinformatics analysis by using the CAZy database and SCHEMA recombination to design gene sequences which will fulfill those conditions resulted in production of a chimeric protein. We derived the following full length DNA sequence (Figure 1) which showed homology to Cel A gene of *A. thermophilum* (Figure 2) and we expressed and purified the recombinant protein by His-tag method (Figure 3). The activity of this novel chimeric protein was determined to be cellulase when tested for activity by standard Park Johnson assay (Table 1). Thus our recombinant chimeric proteins have definite Cellulase enzyme characteristics. We look forward to scaling up productions and temperature and pH stability testing for its usefulness for bioremediation.

**Acknowledgments**

Supported by USA - Department of Energy (DOE) Grant and NIH Grant #T34GM100831 to Dr. Hirendra Nath Banerjee.

**References**

1. Cheng YS, Ko TP, Wu TH, Ma Y, Huang CH, et al. Crystal structure and substrate-binding mode of cellulase 12A from *Thermotoga maritima*. Proteins. 2010; 79:1193–1204. [PubMed: 21268113]
2. Blumer-Schuette SE, Kataeva I, Westpheling J, Adams MW, Kelly RM. Extremely thermophilic microorganisms for biomass conversion: status and prospects. Curr Opin Biotechnol. 2008; 19:210–217. [PubMed: 18524567]
3. Fierobe HP, Bayer EA, Tardif C, Czjzek M, Mechaly A, et al. Degradation of cellulose substrates by cellulose chimeras. Substrate targeting versus proximity of enzyme components. J Biol Chem. 2002; 277:49621–49630. [PubMed: 12397074]
4. Blum DL, Kataeva IA, Li XL, Ljungdahl LG. Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. J Bacteriol. 2000; 182:1346–1351. [PubMed: 10671457]
5. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev. 2002; 66:506–577. [PubMed: 12209002]
6. Boraston AB, Bolam DN, Gilbert HJ, Davies GJ. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J. 2004; 382:769–781. [PubMed: 15214846]
7. Tamaru Y, Doi RH. Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. Proc Natl Acad Sci USA. 2001; 98:4125–4129. [PubMed: 11259664]
8. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, et al. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res. 2009; 37:233–238.

9. Reverbel-Leroy C, Pages S, Belaich A, Belaich JP, Tardif C. The processive endocellulase CelF, a major component of the Clostridium cellulolyticum cellulose: purification and characterization of the recombinant form. J Bacteriol. 1997; 179:46–52. [PubMed: 8981979]

10. Bronnenmeier K, Kundt K, Riedel K, Schwarz WH, Staudenbauer WL. Structure of the Clostridium stercorarium gene celY encoding the exo-1,4-beta-glucanase Avicelase II. Microbiology. 1997; 143:891–898. [PubMed: 9084173]

11. Wang WK, Kruus K, Wu JH. Cloning and DNA sequence of the gene coding for Clostridium thermocellum cellulase Ss (CelS), a major cellulosome component. J Bacteriol. 1993; 175:1293–1302. [PubMed: 8444792]

12. Vazana Y, Morais S, Barak Y, Lamed R, Bayer EA. Interplay between Clostridium thermocellum family 48 and family 9 cellulases in cellulosomal versus noncellulosomal states. Appl Environ Microbiol. 2010; 76:3236–3243. [PubMed: 20348303]

13. Park J, Johnson MJ. A submicrodetermination of glucose. J Biol Chem. 1949; 181:149–151. [PubMed: 15390401]
Figure 1.
Nucleotide sequence of the PCR amplified amplicon.

CATATGGCCACAGCGATGATCCGTATAAGCAACGTTTTCTTGGAACGTGGAAGAGA
GTGGCAGACATCGACCGAACAGGTTATTCTACGTCACCACTGGTATCCGTCGACACCAGC
TGCTCTACTATCTGTGGCCGAGTACCCGTGCATGCAATCGCCGACTACCCGACCGG
CATTGACCCGATTACATGAGCTGTTTGCTCCACTATGTGACCAATACGTATGCGTACCTA
TATGACCTGCGTGTGATTTGAGATGTTGACGACCTGCTGTCGACATCGCCGACCGG
CTCTGATGTATTTCGTGGCGATAGCAGACTCAGCCACTAGGGGAGCGA
TGACGGCATTCAAGCCACCGCCAGAATTGACAGCTCAGCCGGCATGTCGAGGAGC
TACCTCTGCTATGCAATGGTTCGACAAATAACTTTTCGTAATAATTGGTGATTCCAAACAA
GCAGGTACCCGCTAGACCGCGACCCCATATTACCTGTGCTCGTTGCTATAGGGTGCGTGC
GGTGGCCATCACAGGCTATTTGGGCTAGTATGTCGATAGGCTGTTCCACGTTCATTGCGAGGC
TACCAAGATCGGATAGCAGCGGTTGATTCTGGCCCAACAGTCCGGAAGTTAAACCGGA
AAGCCGCAACCGTGCATTGATTTGGGCGAAGGCTGGAGCGCCAGCTGGAGTTTCT
ATCAATGGCTCGACAGAGCTGGCTGATAGCAATCGCCAGGTGTCGACGAGTACGTAC
AAAGGTCCGCTACGAAAACCTCTCCAGACGAGTATCAGACGTTATGCGATCAGCGTAC
GAAGAAACATTTGCTGATCCTTTGACATGCAATCGCCGCTGATTGCTTCCTGACAGC
GGCAGACCTTCGAGATTTCGCCGTCATCCTGGAAATGCTGATACCCGAGCTTTGAC
CCGTACTTACACGGGTAATCAGCACTGCGATGCGGCTGTTTTATTCTAGCTACGG
ACTTGCGGCGACGCGGGCTCTCTGGCAATGCGTCTGCAGTGTATGCACAAACAGCG
GTGACGACGAAGACACGTAAATCTGGCGAAGAAATTTGCTGGACAGTGATTGGGACCTG
TACCGTGACGACAAAGGGTTTTCGTCGACGCCGAGACCTCGCAAAATACGTCCCGCTT
TTGCAAAAGAAGGTCTTACGTTCACAGGGTTGCTCTGTGATACGATGCTACGACTACCG
CGTATCGAACCAGGGTTATTCTTCGACATCCGCTCGAATACTGGAACGAGCG
| Query | Subject |
|-------|---------|
| 1189  | GAAGAACATCCGGGTATACGGATCCCGGATAGCAACAGTGTTGCTTTTCGGCCTTGGTG 1248 |
| 4268  | GAACGGATCCGGATATATATCATAGATCTCTGGGAGCAACACTGTTGATTTTCCGAGTT 4858 |
| 1249  | ACAGATGGGCGGGTGGCGGGAATGATGACTATGACTGACCGGTGATACCGGTGCA-GAGC 1307 |
| 4390  | TCGATGCTACAGAGGCTGAGTGTAGATTACATGGTACGAGGAGAATAGGACGGAGTTAG 4544 |
| 1308  | GTTTGACAAATGGTTGATTGATCAGGGATCTCCGGTTTCTTGACGAGGAGGTACCTAG 1367 |
| 4545  | GCTTGGAGAACTGGGTAGCTGAGTTAAAGGTGATGTGAGATTTGATGTTACGGT 4604 |
| 1368  | CGAGATTTCCCGGTAACCTGGAGTGTCGGGTACCGGACACCTGGAGGTTGACTTACAC 1427 |
| 4605  | TGGATACCGCTGACGCTTGGATTGAGGAGAATACCTGATATGGAACGCGGCTATAC 4664 |
| 1428  | GGTTACTCCGACACCTGGCATGCTACCGGTGTCTTTTATCGTACCGACGCTTGGG 1487 |
| 4665  | AGGAATAGCCAACTATATTCTGAGCAGTTGGACACTTGAGGTGATGTGACTTAC 4724 |
| 1488  | TTTCTCTGGCAACATGCTCTGGTATGATACAAAACACCGGAAAA---TGC------- 1532 |
| 4725  | GTCAATTGGGCAATTTGTTGTTATAGCTGTGACGAGGACGGAGATATGCTGATAG 4784 |
| 1533  | CGACGAAGCAGTAAATCGCTGCGAAAGAATTTCTGAGCTGCAGATAGGTGACCCGGTA 1592 |
| 4785  | TGGAGAGGGCGAGGATTAATTTGCGAGGAATTTCTGCGAACTGAGGTGCTGCAAGG 4844 |
| 1593  | CGACAAAGGGTTTGTCGCCGACCCGAGACTCGCGAGAGTACGTCGGCTCTCTGGAACAAGA 1652 |
| 4845  | TGGAGAGGGTATTGTGACGGCGAGAGGACGGCGACTACGAAAGGTCTCTTTGAGCCAGA 4904 |
| 1653  | GGGTTAAGGTCCACAGGGTTGGTCTGGATGCAAGTACGCTAACCGGAGACGCTGATCGAACC-G 1711 |
| 4905  | GTTATATATATACCGGAGAATGGTAAGGGGAGATCGACCGAATGAGATGTAATAAAAAG 4963 |
| 1712  | GTTTACTTTTCCTGGGACATCCGGCCTGGAAACTCTGGAA-GGCCGGACACTCCGAAGCT 1769 |
Figure 2.
NCBI-BLAST search result of the sequenced amplicon DNA.
Figure 3.
Lane 1=Protein marker, Lane 3–6=Different fractions of bacterial protein expressed, Lane 7–10=His-tag purified recombinant cellulase like Chimeric protein.
### Table 1

Showing cellulase bioactivity of the novel recombinant chimeric protein by Park Johnson Assay.

| Enzyme Concentration | Bioactivity |
|----------------------|-------------|
| 100 µg/µl            | 0.50        |
| 50 µg/µl             | 0.25        |
| 25 µg/µl             | 0.15        |
| 10 µg/µl             | 0.05        |