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Improvement of Cellulase Activity Using Error-Prone Rolling Circle Amplification and Site-Directed Mutagenesis

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Improvement of endoglucanase activity was accomplished by utilizing error-prone rolling circle amplification, supplemented with 1.7 mM $MnCl_2$. This procedure generated random mutations in the *Bacillus amyloliquefaciens* endoglucanase gene with a frequency of 10 mutations per kilobase. Six mutated endoglucanase genes, recovered from six colonies, possessed endoglucanase activity between 2.50- and 3.12-folds higher than wild type. We sequenced these mutants, and the different mutated sites of nucleotides were identified. The mutated endoglucanase sequences had five mutated amino acids: A15T, P24A, P26Q, G27A, and E289V. Among these five substitutions, E289V was determined to be responsible for the improved enzyme activity. This observation was confirmed with site-directed mutagenesis; the introduction of only one mutation (E289V) in the wild-type endoglucanase gene resulted in a 7.93-fold (5.55 U/mg protein) increase in its enzymatic activity compared with that (0.7 U/mg protein) of wild type.

Keywords: Endoglucanase gene, error-prone rolling circle amplification, site-directed mutagenesis, *Bacillus amyloliquefaciens*

Cellulose, the most abundant renewable resource on Earth, is a polysaccharide composed of β-D-glucopyranosyl units joined together by 1,4-glycosidic bonds. The three major types of cellulolytic enzymes are the following: endoglucanases (E.C. 3.2.1.4), exo-cellobiohydrolases (E.C. 3.2.1.91), and β-glucosidases (E.C. 3.2.1.21) [4, 5]. Cellulases play an important role in the generation of ethanol from cellulosic substrates by digesting cellulose and creating fermentable sugars. To date, the cost of producing ethanol with cellulases remains high, as large amounts of cellulase are required for cellulose digestion. Therefore, a reduction in cellulase production costs, an improvement in cellulase activity, and an increase in sugar yields are all vital for reducing the costs of processing bioethanol [10, 20]. Consequently, scientists have used mutagenesis to develop microbial strains with improved cellulase activity.

In vitro random mutagenesis coupled with genetic selection or high-throughput screening is a technique for developing enzymes with novel properties. There are several methods used for random mutagenesis, which include error-prone PCR [14, 19], bacterial mutator strains [10], and rolling circle amplification (RCA) [15, 16]. Rolling circle amplification is an isothermal method that amplifies circular DNA by a rolling circle mechanism [11], yielding linear DNA composed of tandem repeats of the circular DNA sequence. This method has several advantages over conventional methods for amplifying DNA, and these include ease of amplifying circular DNA [6] and the ability to directly transform the RCA product into host cells [8]. Mutagenesis with rolling circle amplication and site-directed mutagenesis are powerful tools for evolving novel enzymes [1, 12], and an improved and more convenient RCA method, error-prone RCA, has been developed [9]. This method consists of only one RCA step followed by the direct transformation of the host strain, and it yields mutants with an adequate mutation frequency for *in vitro* evolution experiments.

The microbe *B. amyloliquefaciens* DL-3 has been previously isolated from the soil based on its ability to hydrolyze rice hulls, and the cellulase produced by the bacterium was purified and characterized [13]. The purified cellulase hydrolyzed avicel, caboxymethylcellulose, cellobiose, beta-glucan, and xylan. The objective of the present study was to improve endoglucanase activity by utilizing error-prone rolling circle amplification (epRCA) in the presence of $MnCl_2$ to generate mutant variants of the endoglucanase

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gene. Mutants generated by this method were then studied through site-directed mutagenesis.

Materials and Methods

Endoglucanase Gene, Plasmid, and Host Cells

The *Bacillus amyoliquefaciens* DL-3 endoglucanase gene (1,500 bp) inserted in pGEM-T (3,015 bp) to create recombinant pGEM-T plasmid containing the endoglucanase gene [13] was obtained from professor Jin-Woo Lee at Department of Biotechnology, Dong-A University, Busan, Korea. Plasmid pGEM-T (3,015 bp) was purchased from Promega Co. (Madison, USA). The *E. coli* strain JM109 with genotype *recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^-, m_k^+) *supE44 relA1* Δ(*lac-proAB*), (F´*tra*D36 *proAB laq*I^{q}ZΔM*15*), and the XL1-Blue strain with genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F´ *proAB lacI*^{q}ZΔ*M15 Tn10* (Tet^r)) were purchased from Promega (Madison, USA) and Stratagene (USA), respectively.

Plasmid Preparation

A pGEM-T harboring the endoglucanase gene was isolated from *E. coli* strain JM109 and purified using a Kit Wizard Plus SVMinipreps DNA Purification System (Promega, Cat. No. A 1460, USA).

Error-Prone Rolling Circle Amplification (epRCA)

A TempliPhi100 Amplification Kit (Code: 25-6400-10) was purchased from GE Healthcare UK Limited (UK). The kit consists of sample buffer, reaction buffer, and enzyme mix. The sample buffer contains random hexamers that prime DNA synthesis nonspecifically, and it is also used to resuspend bacterial cells from a colony, cell culture, or other input DNA. The reaction buffer contains salts and dNTPs and is adjusted to a pH that supports DNA synthesis. The enzyme mix contains Ø29DNA polymerase [6] and random hexamers in 50% glycerol. The epRCA of the endoglucanase gene was performed in the presence of 1.0−3.5 mM Mn^{2+}. This condition reduced the fidelity of DNA amplification at constant temperature, which resulted in many mutant variants of the endoglucanase gene. The resulting DNA was used for the transformation of *E. coli* strain JM109.

A 0.5 µl aliquot containing 2 ng of template (the endoglucanase gene inserted in the pGEM-T) was mixed with 5 µl of sample buffer. The mixture was heated to 95°C for 3 min to denature the plasmid and then cooled immediately to 25°C. The amplification reaction was started by adding a mixture from the TempliPhi Kit consisting of 5 µl of reaction buffer, 0.2 µl of enzyme mix, and 1 µl of $MnCl_2$ solution. Concentrations of $MnCl_2$ ranging from 1.0 to 3.5 mM were used in the epRCA reaction. The mixture was incubated at 30°C for 18 h and subsequently heated to 65°C for 10 min to stop the reaction. The aliquot of epRCA product was purified using the MinElute Reaction Cleanup Kit (Qiagen, Germany; Cat. No. 28204). The size and quantity of amplified DNA were estimated by using 1% agarose gel electrophoresis and a DNA marker (GenePia, Bionics, Korea; Cat. No. D1001-6).

Site-Directed Mutagenesis

A QuikChange II Site-directed Mutagenesis Kit (Cat. No. 200523) purchased from Stratagene (USA) was used in this study. The following two mutagenic oligonucleotide primers were used to generate the desired mutation (E289V) in the wild-type endoglucanase gene (mismatches are in boldface and underlined):

F5'-GCAAGAACATCAGCTGGG**T**GAACTGGAATCTTTCTG-3'
R3'-CAGAAAGATTCCAGTTC**A**CCCAGCTGATGTTCTTGC-5'

Media Preparation

Luria−Bertani agar composed of 1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, and 1.5% agar was autoclaved at 121°C for 15 min and then cooled to 50°C. To this mixture, ampicillin was added to a final concentration of 100 µg/ml (LB/amp), and 20 ml of the medium was then poured into a sterile petri dish. Prior to using a plate, 100 µl of 10 mM IPTG and 100 µl of 2% X-Gal solution were spread over the surface of the (LB/amp) plate and allowed (LB/amp/IPTG/X-Gal) to absorb for 30 min at 37°C.

Transformation

High-efficiency competent cells of *E. coli* strain JM109 purchased from Promega Co. (Madison, USA) were prepared and transformed with 3~4 ng of epRCA product in accordance with the manufacturer's directions. The transformation mixture (100 µl) was plated onto duplicate LB/amp/IPTG/X-Gal plates and incubated at 37°C for 18 h. The white colonies were picked and used for further study of screening and selection of transformants with higher enzyme activity.

Enzyme Assay

The quantity of reducing sugars formed by endoglucanase was determined with 3,5-dinitrosalicylic acid (DNS) using glucose as a standard [17]. The endoglucanase activity was assayed in 0.05 M sodium acetate buffer, pH 4.8, by reaction with 1% sodium-CMC. Five hundred microliters of substrate solution was pre-incubated in a water bath at 50°C for 5 min. To this solution, 0.5 ml of appropriately diluted enzyme was added and incubated at 50°C for 30 min. The enzymatic reaction was then stopped by adding 1 ml of DNS. For the blank, 0.5 ml of enzyme solution was replaced by acetate buffer and mixed with 1 ml of DNS. To this mixture, 0.5 ml of substrate solution was added and mixed well. The reaction and blank solutions were then boiled at 100°C for 5 min, subsequently cooled, and 5 ml of distilled water was added. The reducing sugar content was determined at 540 nm. One unit (U) of endoglucanase activity was defined as the amount of enzyme in 1 ml that liberates 1 µmol of glucose per minute from sodium-CMC.

Selection of Transformants with High Endoglucanase Activity

The white colonies obtained after transformation were inoculated into a 96-deep-well culture plate (BD Biosciences Co. Ltd), each containing 300 µl of LB broth, 100 µg amp/ml, and 0.1% CMC, and incubated at 37°C in an orbital shaking incubator at 180 rpm for 1 day. After cultivation, the culture broths were transferred into 96-deep-well culture plates for measurement of cellulase activity. Each well contained 50 µl of supernatant and 50 µl of 1% sodium-CMC in acetate buffer, incubated at 50°C for 30 min. In the blank, supernatant was replaced by acetate buffer. The reaction was stopped by adding 100 µl of DNS and boiled at 100°C for 5 min, and then cooled. To this, 0.5 ml water was added and mixed well. A 200 µl of reaction solution was transferred to a 96-well-plate using a multi-channeled micropipette. Then measurement of enzyme activity was performed at 540 nm using an Elisa reader (Biomate, Co. Ltd).

After the screening step, 25 putative mutant colonies were selected and grown on LB/amp/IPTG/X-Gal plates, and then transferred to a 24-well cell culture plate. Each well contained 2 ml of LB broth containing ampicillin (100 µg/ml) and 0.1% CMC. To this, colonies

were inoculated and incubated at 37°C on an orbital shaking incubator operated at 150 rpm for 48 h. The cultures were centrifuged for 10 min at 10,000 rpm and the supernatants were used as crude enzyme samples. The colonies with high endoglucanase activity were selected and cultured again for further study.

For flask test of endoglucanase production, the colony of interest that harbored a mutant endoglucanase gene was grown on a LB/amp plate for 48 h, and then one loopful of cells was inoculated into a 250 ml Erlenmeyer flask containing 100 ml of LB broth, ampicillin (100 μg/ml), and 0.1% CMC. The culture was incubated at 37°C on an orbital shaker operating at 200 rpm. After 48 h, the culture supernatant was prepared and used as a crude enzyme source.

DNA Isolation and Sequencing of Mutant Endoglucanase Genes

Mutant variants of the endoglucanase gene originated from one of six selected colonies: rca222, rca595, rca651, rca654, rca655, and rca666. All of the selected colonies exhibited higher than average levels of endoglucanase activity. DNA isolation and sequencing were performed with the original endoglucanase gene for comparison with the mutant variants. The DNA was sequenced using primers T7 and S6.

Purification of Endoglucanase

The culture from the 250 ml Erlenmeyer flask was centrifuged for 10 min at 12,000 rpm, and then ammonium sulfate (65% saturation) was added to 50 ml of crude enzyme solution with constant stirring. The mixture was kept overnight at 4°C, and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was decanted and precipitate was redissolved in 0.1 M acetate buffer (pH 5.5) and dialyzed for desalination. The dialyzed fraction (12 ml) was applied onto a Sephadex G-100 column (2.5 × 8.0 cm) pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 25 ml/h until OD_{280} was less than 0.01, and then washed with the same buffer. Fifteen fractions of 2.0 ml each were collected. The fractions containing highly active CMCase were pooled. All purification steps were carried out at 4°C. The protein concentration was determined using bovine serum albumin as the standard [3].

Computer Analysis

The analysis of sequence data and sequence similarity searches was conducted using the BLAST program from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Corresponding amino acid sequences were determined using the Proteomics and Sequence Analysis tools from http://www.ebi.ac.uk/Tools/emboss/transeq/index.html. Multi-alignment of amino acid sequences or nucleotide sequences was performed using ClustalW2 tools from http://www.ebi.ac.kr/Tools/clustalW2/. A database of protein domains, families, and functional sites was accessed utilizing ScanProsite Tools from http://www.expasy.org/prosite/. Primers for site-directed mutagenesis were designed using http://www.bioinformatics.org/primerx/ to generate the desired mutations based on the cellulase genes of *B. amyloliquefaciens*.

RESULTS AND DISCUSSION

epRCA Combined with $MnCl_2$ Creates Mutant Variants of Endoglucanase

We studied 4,951 positive colonies exhibiting endoglucanase activity different from wild type. The highest number of colonies (2,689, 54.33%) was obtained from epRCA in the presence of 1.7 mM $MnCl_2$ (Table 1). Of these colonies, 951 mutants had endoglucanase activity that differed significantly from wild type.

The results of assays for enzyme activity in all 4,951 colonies showed that the endoglucanase activities of mutant colonies differed significantly between colonies. The endoglucanase activity in some mutant colonies was improved, but many mutants generated by epRCA in the presence of $MnCl_2$, especially at higher $MnCl_2$ levels (> 3.0 mM), had less endoglucanase activity than wild type (data not shown). These results suggest that in epRCA, $MnCl_2$ plays a crucial role in determining the number and variety of mutants.

Among the 4,951 colonies, 25 showed activity between 1.14-fold and 3.18-fold higher than that of wild type (Table 2). The 7 mutants with the highest enzyme activities (> 300%; rca586, rca595, rca651, rca654, rca655, rca666, and rca1947) were found in the mutant variants generated from epRCA in the presence of 1.7 mM $MnCl_2$ (Table 2). The higher activities were confirmed again using the crude enzyme obtained from a larger culture volume of 100 ml. In the selected mutant library of 25 colonies, 64% of the mutant variants were generated from epRCA in the presence of 1.7 mM $MnCl_2$. The findings of this study were similar to those of Fujii *et al*. [9], in which 1.5 mM $MnCl_2$ in the

Table 1. Libraries size of *E. coli* strain JM109 harboring a presumed mutant endoglucanase gene inserted in pGEM-T.

$MnCl_2$ (mM)[a]	Selected colony number[b]	Percent in total number of tested colonies[c]	Percent in total number of selected colonies
1.0	260	17.3	5.3
1.3	375	25.0	7.6
1.7	2,689	89.6	54.3
2.0	764	50.9	15.4
2.5	455	30.3	9.2
3.0	408	27.2	8.2
Total	4,951		100.0

[a] Concentration (mM) of $MnCl_2$ used in epRCA reaction.

[b] The total number of colonies examined for each concentration of $MnCl_2$ was 1,500, except for 1.7 mM $MnCl_2$ (3,000 colonies), and the numbers of colonies with higher enzyme activity than that of wild type are shown here.

[c] The percent of selected colony number in total number of colonies used for enzyme activity analysis, for each concentration of $MnCl_2$.

Table 2. Improved enzyme activity of colonies harboring mutant endoglucanase genes.

Colony no.[a]	$MnCl_2$ (mM)[b]	Endoglucanase activity (U/ml)	Activity (mean, %)[c]	Percent in total number of selected colonies
Wild type	0.0	10.60	100[d]	
rca222	1.3	27.36	258.12	4
rca289	1.7	25.68	242.30	
rca586	1.7	33.13	313.53	
rca595	1.7	32.45	306.12	
rca651	1.7	33.44	315.45	
rca654	1.7	33.73	318.17	
rca 655	1.7	32.76	309.10	
rca666	1.7	32.76	309.10	
rca716	1.7	28.43	268.18	64
rca845	1.7	25.78	243.20	
rca1947	1.7	31.81	300.10	
rca2183	1.7	26.84	253.20	
rca337	1.7	25.54	240.91	
rca677	1.7	24.87	234.60	
rca836	1.7	23.13	218.18	
rca1850	1.7	22.16	209.09	
rca2834	1.7	25.05	236.36	
rca653	2.0	27.05	255.20	
rca1922	2.0	14.18	133.82	
rca2297	2.0	12.10	114.12	20
rca1427	2.0	15.90	150.00	
rca1611	2.0	15.90	150.01	
rca2524	2.5	28.11	265.21	4
rca1133	3.0	27.95	263.64	8
rca1157	3.0	15.12	142.65	
Total				100

[a]Only the levels of endoglucanase activity from high-activity colonies are presented here.
[b]Concentration (mM) of $MnCl_2$ in epRCA reaction.
[c]Single colony was cultivated in 24-well cell culture plates, each well containing 2 ml of LB broth containing 100 μg ampicillin/ml and 0.1% CMC and incubated at 37°C on an orbital shaking at 125 rpm. After cultivation for 48 h, the enzyme activity was measured. Three replicas of each mutant colony were cultured using the same scale and culture conditions.
[d]100% activity was 10.6 U/ml (0.7 U/mg protein).

epRCA reaction was suitable to generate mutations on the TEM-1 β-lactamase gene. In agreement with that result, we found that both low concentrations (<1 mM) and high concentrations (>3 mM) of $MnCl_2$ in the epRCA reaction generated a lower mutation frequency (data not shown). The lower mutation rate, due to the poor frequency of mismatch incorporation during epRCA, seemed to be related to a lower concentration of $MnCl_2$. To overcome this problem, either an increased concentration of $MnCl_2$ or a decreased concentration of template DNA in the epRCA reaction can be used. Levels of $MnCl_2$ in the epRCA reaction were kept below 2 mM, since excess $MnCl_2$ decreases epRCA yields [7]. However, the use of a modified Phi29 DNA polymerase (H61R) without 3'-5' exonuclease activity may increase the mutation frequency and epRCA yield [7]. The effect of $MnCl_2$ on enzyme activity has been documented [2, 14].

DNA Sequencing and the Nature of the Mutations on the Endoglucanase Gene

We selected six clones, rca222, rca595, rca651, rca654, rca655, and rca666 (five clones from the reaction with 1.7 mM and one clone from the reaction with 1.3 mM), that produced higher levels of endoglucanase than wild type. The mutant variants of the endoglucanase gene were recovered from the six selected clones and, along with one wild-type clone, were sequenced and identified. The mobility of the recovered plasmids on a 1% agarose gel was essentially identical to that of the wild-type endoglucanase gene (data not shown).

The distribution of the mutations is shown in Fig. 1. Mutations were only found between nucleotides 1 and 205 and nucleotides 850 and 1497. Multi-alignment between the mutant and wild-type endoglucanase genes was performed using ClustalW2 (http://www.ebi.ac.uk). The mutant variations of the endoglucanase gene are shown in Table 3. The endoglucanase genes of the six recovered plasmids had the different nucleotide mutations on the genes, but showed the same six mutated amino acids: A15T, P24A, P26Q, G27A, E289V, and H499N (Table 3 and Fig. 2). In addition to these six mutations, variant N279I was only found in the sequence from clone rca666 (Table 3 and Fig. 2).

Identification of Mutant Endoglucanase Genes

The mature *B. amyloliquefaciens* endoglucanase gene contains 1,497 nucleotides and 499 amino acids [13]. Most cellulases, such as xylanase and β-1,3-1,4-glucanase, are modular enzymes consisting of two or more functional modules: catalytic and carbohydrate-binding modules

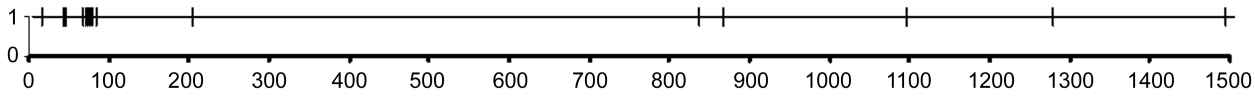

Fig. 1. Distribution of mutations in the endoglucanase gene.
Six mutant clones generated by epRCA were sequenced.

Fig. 2. Clustal 2.0.10 sequence alignment of the mutant and wild-type amino acid sequences.
Origin: Original endoglucanase gene sequence of *B. amyloliquefaciens* DL-3. rca654, rca651, rca222, rca595, rca666, and rca655: Mutant variants of the endoglucanase gene sequence generated by epRCA.

(CBMs) connected to each other *via* a linker sequence [18]. Cellulase from *B. amyloliquefaciens* DL-3 consists of a catalytic domain (Gly-31 to Asn-332) of the glycoside hydrolase family 5 (GH5) and a family 3 CBM (Glu-350 to His-499). Like the modules (GH5/CBM3) of many *Bacillus* endoglucanases, the DL-3 enzyme has its catalytic domain in the N-terminal portion and its CBM in the C-terminal portion. Based on our analysis of the mutated endoglucanase gene, the E289V mutation (Table 3 and Fig. 2) could be the most important amino acid for cellulase catalytic activity since it is located in the cellulase catalytic domain. Other variant sites are located in other regions such as the linker and the cellulose-binding domain.

Site-Directed Mutagenesis Creates the Mutant Variant E289V

To confirm that the variant E289V was the amino acid responsible for the improved activity of cellulase, site-directed mutagenesis was used. Site-directed mutagenesis

Table 3. Nucleotide changes in mutant genes by error-prone rolling circle amplification.

Clone no.	Nucleotide change	Amino acid substitution	Clone no.	Nucleotide change	Amino acid substitution	Clone no.	Nucleotide change	Amino acid substitution
222	GCT→ACT	A15T	595	GCT→ACC	A15T	651	GCT→ACG	A15T
	CCT→GCC	P24A		CCT→GCT	P24A		CCT→GCT	P24A
	CCC→CAG	P26Q		CCC→CAG	P26Q		CCC→CAA	P26Q
	GGT→GCG	G27A		GGT→GCC	G27A		GGT→GCC	G27A
	GAG→GTG	E289V		GAG→GTT	E289V		GAG→GTC	E289V
	CAT→AAT	H499N		CAT→AAT	H499N		CAT→AAC	H499N
654	GCT→ACA	A15T	655	GCT→ACA	A15T	666	GCT→ACC	A15T
	CCT→GCG	P24A		CCT→GCT	P24A		CCT→GCC	P24A
	CCC→CAG	P26Q		CCC→CAA	P26Q		CCC→CAA	P26Q
	GGT→GCA	G27A		GGT→GCC	G27A		GGT→GCA	G27A
	GAG→GTG	E289V		GAG→GTA	E289V		AAT→ATC	N279I[a]
	CAT→AAC	H499N		CAT→AAC	H499N		GAG→GTG	E289V
							CAT→AAT	H499N

[a]The mutant N279I was only found in this clone (rca666).

technique replaced the glutamic acid at position 289 with a valine. As a result, the host cell harbored a mutation at position 289, and its enzyme activity increased more than 7.93-fold (5.55 U/mg protein) over wild type (0.7 U/mg protein). This observation demonstrated that E289V was the amino acid responsible for the improved cellulase activity.

In conclusion, the present study utilized epRCA in the presence of 1.7 mM $MnCl_2$ to generate random mutations in endoglucanase genes inserted in pGEM-T. Some of these mutants had endoglucanase activity between 1.14- and 3.18-fold higher than wild type. Variant E289V, which is located in the catalytic domain of the endoglucanase gene, was an important amino acid for this improved activity. Therefore, we used mutagenic primers and site-directed mutagenesis to generate the E289V mutation in the wild-type endoglucanase gene. The activity of endoglucanase generated in this way, with V (valine) at position 289, was 7.93-fold higher than that of wild type.

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