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 MnCl₂. 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
gene. Mutants generated by this method were then studied through site-directed mutagenesis.

**Materials and Methods**

**Endoglucanase Gene, Plasmid, and Host Cells**

The *Bacillus amyloliquefaciens* 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 (rK-m-, mK-) supE44 relA1 M(lac-proAB), (F' traD36 proAB lacQZAM15), and the XL1-Blue strain with genotype recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacZAM15 Tn10 (Tet)) 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 SV Minipreps 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 O29DNA polymerase [6] and random hexamers in 50% glycerol. The epRCA of the endoglucanase gene was performed in the presence of 1.0–50% glycerol. 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 MnCl₂ solution. Concentrations of MnCl₂, 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 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 µl of enzyme solution was replaced by acetate buffer and mixed with 1 ml of DNS. To this mixture, 0.5 µl 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 plates for measurement of cellular 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 solution reaction 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).

**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 µl of enzyme solution was replaced by acetate buffer and mixed with 1 ml of DNS. To this mixture, 0.5 µl 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.