Cloning of Cellobiohydrolase Gene (*cbhI*) in Radiation Induced Mutant of *Pleurotus florida*

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Abstract - The cellobiohydrolase gene (*cbhI*), a key component of a cellulolytic system, of a mutant PfCM4 (*Pleurotus florida*), developed through gamma ray radiation mutagenesis, was isolated and cloned. The deduced amino acid sequence was closely related to the glycoside hydrolase family 7 (GH7). The molecular weight of the deduced amino acid sequence of *cbhI* gene was found to be 22.4 kDa. Though the percent identity was found to be much less (35.61%) between the wild type and mutant, the cellulolytic activity of PfCM4 was 17.24% higher than that of the wild type. This shows that the catalytic domain of the *cbhI* gene was conserved in the mutant PfCM4.

Key words : Cellobiohydrolase, Mutant, *Pleurotus florida*, Radiation

INTRODUCTION

Lignocellulosic biomass such as agro-forestry-industrial residues, recognized as a potential sustainable source for biofuels and biomaterial production, are principally made up of cellulose (40～50%), hemicelluloses (25～30%), and lignin (15～20%) (Knauf and Moniruzzaman 2004). The potential utilization of all three components will play a significant role in economic viability of the lignocellulosic biomass for value added products. Cellulose, a linear polysaccharide consisting of β-1,4-linked D-glucopyranosyl units, is the major component of plant material (20～40%) and the most abundant biomass on earth (Tomme *et al*. 1995, Zhao *et al*. 2012). At least, three major types of cellulase activities are believed to be involved in cellulose hydrolysis: Endo-β-1,4-glucanases (EC 3.2.1.4) hydrolyze cellulose chains in a non-processive (random) manner, whereas exoglucanases such as cellodextrinases (EC 3.2.1.74) or cellobiohydrolases (EC 3.2.1.91) depolymerize cellulose chains from their reducing or non-reducing ends in a processive or ordered manner. β-Glucosidases (EC 3.2.1.21) cleave cellooligosaccharides (especially cellobiose) to liberate glucose (Ni and Tokuda 2013). The cellobiohydrolase (CBH) enzymes are known to be the key component of a cellulose system and the major product of their action on cellulose is cellobiose (Teeri 1997). Mushrooms can degrade virtually any lignocellulosic waste and play a vital role in disposing a lot of waste including domestic, industrial, and agricultural waste (Okhuoya 2011).Valaskova and Baldrian (2006) reported that a lignocellulose-degrading enzymatic system is of major importance for substrate colonization and carbon acquisition by wood-rotting and soil saprophytic fungi. Mushroom cultivation is a highly efficient method of disposing agro-lignocellulosic waste as well as producing nutritious food (Cohen *et al*. 2002), and this in turn can minimize the environmental pollution owing to unutilized agricultural waste as well as reduce air pollution associated with the burning of agricultural waste (Ahmed *et al*. 2009). Mutation breeding is a process by which mutant variants with desirable traits to be bred are developed, without altering the remain-

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ing genotype using physical, chemical and biological mutagenic agents (Sathesh-Prabu and Lee 2011). In a previous study, we developed a potent cellulase mushroom mutant of *Pleurotus florida*, designated as PfCM4, induced by gamma radiation at a dose of LD_{99} (0.51 kGy) (Sathesh-Prabu and Lee, 2012) which showed 17.24% more cellulolytic activity than a wild type ($p < 0.05$), and the genetic similarity between wild type and PfCM4 was found to be 22.30%, as determined by amplified fragment length polymorphism (AFLP). In addition, mycelial and secreted protein profiling by 2D-electrophoresis analysis showed at least three and five different protein spots in the range of 25 kDa to 100 kDa, respectively, in PfCM4. In the present study, we report the cloning and sequence analysis of the CBH gene from wild and gamma ray induced mutant strains of *P. florida* (PfCM4).

MATERIALS AND METHODS

1. Strain and chemicals

The pure culture of *Pleurotus florida* (KACC 52110) was procured from the Korean Agricultural Culture Collection Centre, Suwon, Korea. The wild and mutant cultures were maintained in a potato dextrose agar (PDA) medium at 4°C and sub-cultured at 30-day intervals. All chemicals and microbiological media were purchased from Sigma-Aldrich and Difco, respectively. All chemicals used were of analytical grade unless otherwise stated.

2. DNA extraction

DNA extractions were performed using a standard CTAB method with slight modifications. Fresh mycelia of wild type and PfCM4 (0.1 g) were collected from 7-day old cultures grown in potato dextrose broth through centrifugation at 12,000 rpm for 10 min and frozen with liquid nitrogen. Frozen mycelia were ground with a sterile mortar and pestle and kept in a 1.5 ml micro-tube. A preheated (65°C for 30 min) 2X CTAB extraction buffer (700 µl) was added to each of the micro-tubes and the mixture was incubated at 65°C for 30 min; 700 µl of Chloroform : Isoamyalcohol (24 : 1) was then added followed by centrifugation at 14,000 rpm for 30 min at 4°C. Afterwards, the top aqueous upper phase was transferred to a 1.5 ml micro-tube and two volumes of 95% alcohol were added and incubated on ice for 10 min followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. Subsequently, the collected pellet was washed with 70% alcohol, then air dried, and the DNA pellet was resuspended in a 200µl of TE buffer. The purity and concentration of the extracted DNA were estimated through spectroscopy (Nanodrop-ND1000, Nanodrop Tech. Inc., USA), and the quality of the DNA was obtained by means of electrophoresis in 1% agarose gels, followed by staining with ethidium bromide. A DNA stock solution was kept at −20 °C for further analyses.

3. Cloning and sequence analysis of CBH

To amplify the CBH region from the wild type and PfCM4, degenerate primers employed by Chukeatirote *et al*. (2012) (CBH_FP: 5′-TGYGAYTCICARTGYCCICGIGA-3′ and CBH_RP: 5′-GARTCIAGCCAIAGCATRTTIGC-3′) were synthesized (Macrogen, Korea) and used for PCR reactions on a thermal cycler (MJ Research Inc., Korea) with the following conditions: 35X (94°C for 30 s, 52°C for 30 s and 72°C for 1 min). After ascertaining the presence and amplification of CBH on agarose gel, the amplicons from the gel were purified (GeneAll, Korea) and cloned using a TA cloning vector system (RBC, Korea). The plasmids were transformed into a DH5α cell (Hit competent cell, RBC, Korea). The positive clones were selected using blue-white screening method and the plasmids were extracted (GeneAll, Korea) for CBH gene nucleotide sequencing (Macrogen, Korea). The nucleotide sequences were analysed using the ClustalOmega and BLAST programmes.

RESULTS AND DISCUSSION

Cellobiohydrolases are classified into three glycoside hydrolase (GH) families, i.e., GH6, GH7 and GH48, based on their amino acid sequence similarity (Henrissat 1991). Among three families, only GH7 is thought to be exclusively fungal, and contains CBHI cellobiohydrolase and endogluconases from both ascomycete and basidiomycete fungi (Edwards *et al*. 2008). Owing to the low activity of Endo-β-1,4-glucanases (EC 3.2.1.4) to hydrolyze crystalline cellulose, CBH is considered to be an important component in hydrolyzing crystalline cellulose (Hong *et al*. 2003). Genomic DNAs of wild type and PfCM4 were used as a template

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Ref.        GAATCGAGCCAGAGCATGTTGGCGGCGTGATCACTCCAGAGGGACATGATGAGGACCATG 60
Wild        GAATCGAGCCAGAGCATATTGGCGGCGTGATCACTCCAGAGGGACATGATGAGGACCATG 60
PfCM4       GAATCGAGCCAGAGCATGTTGGCGGCGTGGTCGTCCCAAACGGACATGACCAGGACCATG 60
            ***************** ********** **   *** * ********  *********

Ref.        CCATTATCGAAGGCATCGCCCATCTTCGCCGTAGCACCGTGGGTGTTGTAGTACTGGTTG 120
Wild        CCATTGTCGAAGGCGTCGCCCATCTTCGATGTGGCACCGTGGGTGTTGTAGTACTGGTTG 120
PfCM4       CCGTTGGCGAAGGCGTCACCCATCTTGGCTAATCCGCCCTTGGAGGCGAACGAGTTGGTG 120
            ** **  ******* ** ******** *      * ** * ** *  * *  * * * **

Ref.        TCGCCGAACAGAGTCTTCTGGGCGACACAGAACTCATCAGAGATGGAGTTGTACTGAGAT 180
Wild        TCGCCGAACAGGGTCTTTTGGGCGACACAGAAATCATCAGAGATGGAGTTGTACTGCGAC 180
PfCM4       TCGCCGAAGGCGGTCTTCTGGGCAGCGCACCAGCTGTCGGTGATGGAGTT-TCCGTCAGT 179
            ********    ***** *****  * **  *    ** * ********* * *

Ref.        AACGAGGG-GAAGGTGGAGAATGAGTTCTGGTAGACAACGCCATTCTGGACGTAAACACG 239
Wild        AATGATGG-GAAGGTGGAGAAAGAGTTCTGGCGGACAACACCATTCTGAACATAAACGCG 239
PfCM4       AAGGCTGGCGATGTTGGTCTTGGAGTTCTGGTAGACGACGCCATTCTGGACGTAGATACG 239
            ** *  ** ** * ***     *********  *** ** ******** ** ** *  **

Ref.        GCGGATCTCGACAAGGTTGCCGGTGGGAGTGTTGTTGTCAGTAATGAACTGAGTCACGAC 299
Wild        GCGGATCTCGACGAGGTTGCCGGTGGGGGTGTTGTTGTCAGTGATGAACTGAGTCACGAC 299
PfCM4       GCGAATCTCGGAGAGTTTGCCAGTTGCGGAACCATTGTCGGTGATGAAC--------AAC 291
            *** ******   ** ***** ** *  *     ***** ** ******         **

Ref.        AGTCACCTTCTTCGTGGTGTCGATTGTCTTGCCGCGGCCGAGGAAGTTCTTGTCACCCAT 359
Wild        AGTGATCTTCTTGGTAGTATCGATTGTCTTGCCGCGCCCGAGGAAGTCCTTGTCACCCAT 359
PfCM4       TGTAATCTTCTTGGTCGTGTCGATGGTCTTTCCTTTTCCGAGGAAGTTCTTGTCGCCCAT 351
             ** * ****** ** ** ***** ***** **    ********** ****** *****

Ref.        GCGATAAGAGTTGAAGTCGCAGCCATCCTTGTCACAGACGCCTCCGTAGCGGTTGTTGCC 419
Wild        GCGGTAAGAGTTGAAGTCACAACCATCCTTGTCGCAGACGCCGCTATAGCGGTTGTCTCC 419
PfCM4       GCGGAAGGAGTTGAAGTCACAACCATCTTTGTCGCAAACACCGCCGTATCTGTTGTCGCC 411
            ***  * *********** ** ***** ***** ** ** ** *  ** * *****  **

Ref.        ATCACCACACTCGGTGCCCGAGCAGCGGTAGAGACCCTCGTCACGGCAGACG--TGAGGA 477
Wild        ATCACCGCACTCAGTGCCAGAGCAGCGGTAGAGACCCTCATCGCGACAGACG--TGAGGG 477
PfCM4       ATCACCGCATTCGGTCCCGGAGCAGCGACGG--ATCCCAGTTGCGCTACACGGGTGCGGG 469
            ****** ** ** ** ** ********   *  * **   *  **  * ***  ** **

Ref.        GTGTAGGCAGTGGCTTCAGAGTTAGCCTCCCAGATGTCCATCTCAGCGCAGCACGCTCCG 537
Wild        GTGTAGGCAGTGGCTTCAGAGTTAGCTTCCCAGATGTCCATCTCCGCGCAGCATGCTCCA 537
PfCM4       GTGTATGCAGAAGCCATTGAGTTGGCTTCCCAAATGTCCATCTCACTGCAGCATGCACCG 529
            ***** ****  **    ***** ** ***** ***********   ****** ** **

Ref.        TATCGGCCATTGCCAGCGTTGTCATCAGTCGCAGAAGCGGACCAGTCGAGAACGTTG-GC 596
Wild        TATCGGCCATTGCCAGCGTTGTCATCAGTCGCAGAGGCGGACCAGTCGAGAACGTTG-GC 596
PfCM4       TAGCGACCAGTACCAGCATTAGCGTCGTTGGGGGAACCAGCCCAGTCAAGGACATTATAC 589
            ** ** *** * ***** **  * **  * *  **  * * ****** ** ** **   *

Ref.        CTCGCCATTGATCCACTTGATATCCCGCGGACATTGCGAGTCAC- 640
Wild        CTCTCCGTTGATCCACTTGATGTCCCGCGGAC------------- 628
PfCM4       CTCGCCGTTGATGAACTTGATATCCCGCGGACATTGCGAGTCACA 634
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Fig. 1. Alignment of nucleotide sequences from the *cbhI* gene of the wild type and PfCM4.

to amplify the *cbhI* gene and the expected amplicon (700～800 bp) was eluted, cloned, and sequenced. By submitting the obtained DNA sequence as a query to the BLASTN search of the GenBank database, the results showed a homology (84～88%) toward the *cbhI* gene sequences, especially the glycoside hydrolase family 7 (GH7), of other mushroom fungi, including *P. ostreatus*, *P. sajor-caju*, *P. eryngii* and *Auricularia fuscosuccinea*. After removal of introns from

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Ref.        DSQCPRDIKWINGEANVLDWSASATDDNAGNGRYGACCAEMDIWEANSEATAYTPHVCRD 60
Wild        DSQCPRDIKWINGEANVLDWSASATDDNAGNGRYGACCAEMDIWEANSEATAYTPHVCRD 60
PfCM4       AMSAGYQVHQRRGIMSLTGLVPPTT-----LMLVLVATVHAAVRWTFGKPTQWLLHTPRT 55
             ..  :::  .*  .: .  ..:*          .. ..  :  : .:.* :  *. *

Ref.        EGLYRCSGTECGDGNNRYGGVCDKDGCDFNSYRMGDKNFLGRGKTIDTTKKVT---VVTQ 117
Wild        EGLYRCSGTECGDGNNRYGGVCDKDGCDFNSYRMGDKNFLGRGKTIDTTKKVT---VVTQ 117
PfCM4       R--VAQLGSVAAPGPNAVMATTDTAVFATKMVVTSTPSAWATRTSSEKERPSTRPRRLQL 113
               .      *: .. * *   .. *.     :    .  .  .  .: :. :  *   :

Ref.        FITDNNTPTGNLVEIRRVYVQNGVVYQNSFSTFPSLSQYNSISDEFCVAQKTLFGDNQYY 177
Wild        FITDNNTPTGNLVEIRRVYVQNGVVYQNSFSTFPSLSQYNSISDEFCVAQKTLFGDNQYY 177
PfCM4       FITDNGSATGKLSEIRRIYVQNGVVYQNSKTNIASLTDGNSITDSWCAAQKTAFGDTNSF 173
            *****.:.**:* ****:********** :.:.**:: ***:*.:*.**** ***.: :

Ref.        NTHGATAKMGDAFDNGMVLIMSLWSDHAANMLWLDS 213
Wild        NTHGATAKMGDAFDNGMVLIMSLWSDHAANMLWLDS 213
PfCM4       ASKGGLAKMGDAFANGMVLVMSVWDDHAANMLWLD- 208
             ::*. ******* *****:**:*.**********
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An * indicates positions which have a single, fully conserved residue. A : indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. A . indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

Fig. 2. Alignment of amino acid sequences from the *cbhI* gene of the wild type and PfCM4.

Table 1. Percent identity matrix of *cbhI* of mutant PfCM4

Strain	Ref. strain		Wild type		Mutant-PfCM4	
	nt.	aa	nt.	aa	nt.	aa
Ref. strain	100	100				
Wild type	91.40	100	100	100		
Mutant-PfCM4	74.24	35.61	73.91	35.61	100	100

nt; nucleotide sequence, aa; amino acid sequence

the *cbhI* gene sequence, they were aligned using the Clustal 2.1 program (Fig. 1). The highest BLAST hit (88% similarity) shown by *P. ostreatus* (JQ717253) was considered as the reference strain.

The amino acid sequences of the intron-removed *cbhI* nucleotide sequence were obtained using an online Expasy translate tool, and BLASTP search was performed. Alignment of the deduced amino acid sequence of the wild type-*cbhI* with cellulase genes of other mushroom fungi showed the highest sequence similarities to *cbhI*, especially from *P. ostreatus* (AFJ04532.1, 100%), *A. fuscosuccinea* (AFJ04528.1, 100%), *P. florida* (CAK18800.1, 98%) and *P. eryngii* (AFJ 04530.1, 93%). The sequence similarity between the wild type and *P. florida* (CAK18800.1) showed that the amplified gene belonged to cbhI-2 family of *cbhI*. On the other hand, the amino acid sequence of PfCM4 was less similar to the *cbhI* of *P. ostreatus* (AFJ04532.1, 66%), *A. fuscosuccinea* (AFJ04528.1, 66%), *P. florida* (CAK18800.1, 65%) and *P. eryngii* (AFJ04530.1, 62%). Alignment of the amino acid sequences is given in Fig. 2. The calculated *cbhI* protein molecular weight of the wild type and mutant were found to be 23.6 and 22.4 kDa. It seems that the cellulose binding domain and linker region were not amplified, resulting in a reduction of the molecular weight when compared to that of *P. florida* (CAK18800.1), which has 55.5 kDa of *cbhI*. No signal peptide sequence was predicted by the SignalP online program. However, the percent identity of the amino acid sequences between the wild type and mutant PfCM4 was found to be 35.61% (Table 1). Although, the percent identity was found to be very less between the wild type and mutant, the cellulolytic activity of PfCM4 was 17.24% higher than that of the wild type (Sathesh-Prabu and Lee, 2012). This shows that the catalytic domain of the *cbhI* gene was conserved in the mutant PfCM4.

To conclude, the gamma irradiation of mycelia of *P. florida* could increase the cellulolytic activity of the mushroom. The effect of irradiation on the *cbhI* gene involved in the cellulose hydrolysis was envisaged by the cloning and sequence analysis of *cbhI* gene. The molecular cloning and heterologous expression of the *cbhI* gene of PfCM4 in addition to proteomics study could clearly elucidate the effect of gamma irradiation on the improved cellulolytic activity of the mushroom strain. In addition, further studies will focus on the complete characterization of the cellobiohydrolase protein and increasing the cellulolytic activity much higher by engineering the mutant mushroom at the genomic level for better employment of the mushroom in view of high yield and potential utilization of cellulosic wastes.

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