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Gamma Radiation Induced Mutational Spectrum of Laccase Gene in *Pleurotus ostreatus*

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Abstract - To investigate the mutational spectrum of laccase (Lac) genes (*lac*-A and *lac*-B) involved in degrading lignin which is the recalcitrant cell wall polymer, the genes of the *Pleurotus ostreatus* mutants induced by gamma ray radiation were amplified by PCR and were cloned. All partial *lac*-A genes of 4 mutants (PO-6, -7, -14 and -15) consisted of 1763 base pairs due to the deletion of two bases (491-nt and 492-nt) and addition of one base (875-nt) in 1764 base pairs of *lac*-A gene of PO-1. Totally 36 mutational hot spots were detected and 32 positions were mutated in all of those 4 mutants simultaneously. These mutations were predominantly A : T → G : C transitions (40%). Putative amino acid sequences of *lac*-A genes of mutants have one simultaneous mutated residue (from Thr-44 to Ala-44). The 1764 bp of partial *lac*-B gene was cloned only in PO-5 mutant and contained 19 mutated bases. These mutations were predominantly G : C→A : T transitions (45%). *Lac*-B protein of PO-5 has two mutated residues of Glu-290 and His-363 from Ala-290 and Phe-363, respectively. The hyper-mutational positions were concentrated in specific regions of between 50-nt and 900-nt in *lac* genes. These results suggested that the mutational hotspots responded to gamma radiation could be in some genes, at least *lac*-A and *lac*-B of *P. ostreatus*.

Key words : Gamma radiation, laccase, Mutation spectrum, *Pleurotus ostreatus*

INTRODUCTION

Gamma ray radiation causes a variety of types of damage to DNA in cells (Hutchinson 1985), requiring the concerted action of a number of DNA repair enzymes to restore genomic integrity (Thacker 1999). It has been estimated that upon irradiation of cells, half of the DNA damage caused by direct energy disposition in the cellular DNA (the direct effect) and the other half of the DNA damage resulted from reactions with reactive radicals formed in the vicinity of DNA (the indirect effect) (Michaels and Hunt 1978; Sonntag 1987). The indirect effect of ionizing radiation on DNA could be studied very well in vitro by irradiation of diluted aqueous DNA solutions. Under these circumstances virtually all radiation energy is absorbed by water, leading to the formation of a number of water-derived radicals of which · OH and · H are the most important DNA-damaging species. Therefore, in vitro studies may provide more information on the mutations induced by · OH and · H radicals. From such studies, it seemed that base pair substitutions were the main type of radiation-induced mutations due to the indirect effect and that there was a very strong preference for mutational events at C : G base pairs in the *lacI* and *lacZ* genes of *Escherichia coli* (Sonntag

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1987; Wijker *et al.* 1996).

The information which in vitro studies can provide about the indirect effect of gamma ray radiation, could be possibly too simple models to apply for the in vivo situation. Many other factors could play a role in the damaging process when DNA is present in a cellular environment, such as the direct effect, the generation of other (organic) radicals and the induction of a number of different defense responses. Several studies have been shown that the gamma ray radiation induced mutant frequency of *lacI* gene as a mutational target could increased in *E. coli* (Sonntag 1987; Sargentini and Smith 1994a, b; Wijker *et al.* 1996) and in *lacI* transgenic mice (Winegar *et al.* 1994; Hoyes *et al.* 1998). In hamster ovarian cell (CHO), the majority of *aprt*- and *hprt*-deficient mutants induced by gamma ray radiation differed as transversions and deletions, respectively (Thacker 1986; Miles and Meuth 1989). It has been estimated that the mutation spectrum of cellular DNA induced by gamma ray radiation could be dependant on the target gene loci. Indeed, gamma ray radiation induce not only the deficient mutants (Liwicki 1985; Zolan *et al.* 1988), but also the enhanced mutants (Boominathan *et al.* 1990; Lee *et al.* 2000) in mammalian cells and fungi. And the genetic diversity also increased in the gamma ray radiation induced mutants resulted from RAPD analysis (Lee *et al.* 2000). Therefore, it is necessary to get some more information of the mutation spectrum of several genes from the gamma ray radiation induced individual mutant to analyze precisely in vivo mutation spectrum of gamma ray.

Lignocellulosic materials are the main portion of the biowastes which are abundantly produced by the agricultural industry and forestry stations all over the world. So far, there have been many kinds of researches to recycle or reuse these as useful products, but it was not so successful because of the complexity and recalcitrant nature of lignocellulosic materials. These materials are degraded by the white-rot fungi including edible mushrooms (Leonowicz *et al.*, 1999). Manganese peroxidase (Mnp) and laccase (Lac) of *Pleurotus ostreatus* (oyster mushroom) are the main enzymes to degrade lignin which is the most difficult fraction to be destroyed (Asada *et al.* 1995; Zhao and Kwan 1999; Giardina *et al.* 1995, 1999). By gamma ray radiation the enhanced mutants of ligninolytic ability were induced and characterized, previously, from *P. ostreatus* (Lee *et al.* 2000). These mutants could be the useful strains for the degradation of biowastes and have the difference in genetic similarity. The present study has been carried out to investigate the gamma ray radiation effects on the genomic sequences of *lac* genes of these *P. ostreatus* mutants.

MATERIALS AND METHODS

1. *Pleurotus ostreatus* and its radiation mutants

Mycelia of *P. ostreatus* PO-1 (isolated in Kang-Won province, Korea, KCTC 16812) and its mutants (PO-5, -6, -7, -14, -15 and -16) induced by gamma ray radiation (Co^{60}, 60,000 Ci of capacity, AECL) were cultured in PDB medium according to the previous study (Holm and Berry 1970; Lee *et al.* 2000). The mutants of PO-5, -6, -7 were induced with 1 kGy of gamma ray radiation, and PO-14 was induced with 2 kGy. The PO-15 and PO-16 mutants were derived from PO-14 after re-irradiated with 1 kGy of gamma ray radiation.

2. Cloning and sequencing of *lac* genes

Genomic DNAs were extracted according to Graham (1994) and the polymerase chain reactions (PCRs) were carried out with AccuPower PCR PreMixTM (Bioneer Co., Korea) in 50 *l* of reaction solution containing 20 ng of genomic DNA and 5 pmol of each primers. Specific primers for *lac* gene of *P. ostreatus*, 5′-ACC GCA TCC CCT CAG CCG AT-3′ (LAC-A1, forward), 5′-GAG ATT GAT GTT GAT ATC GG-3′ (LAC-B1, reverse) and 5′-CAA TGC TTT CAA TGG CGA GG-3′ (LAC-B2, reverse) were retrieved from database of POAJ5017 (Giardina *et al.* 1995). All PCR reactions were subjected to the initial denaturation at 94°C for 5 min. These were then processed through 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. These cycles were followed by a single cycle of 5 min at 72°C using Gene Amp PCR System 2400 (Perkin Elmer). Aliquots of the amplified DNA were separated by electrophoresis on 0.8% agarose gel at 10 V/cm. The PCR products were

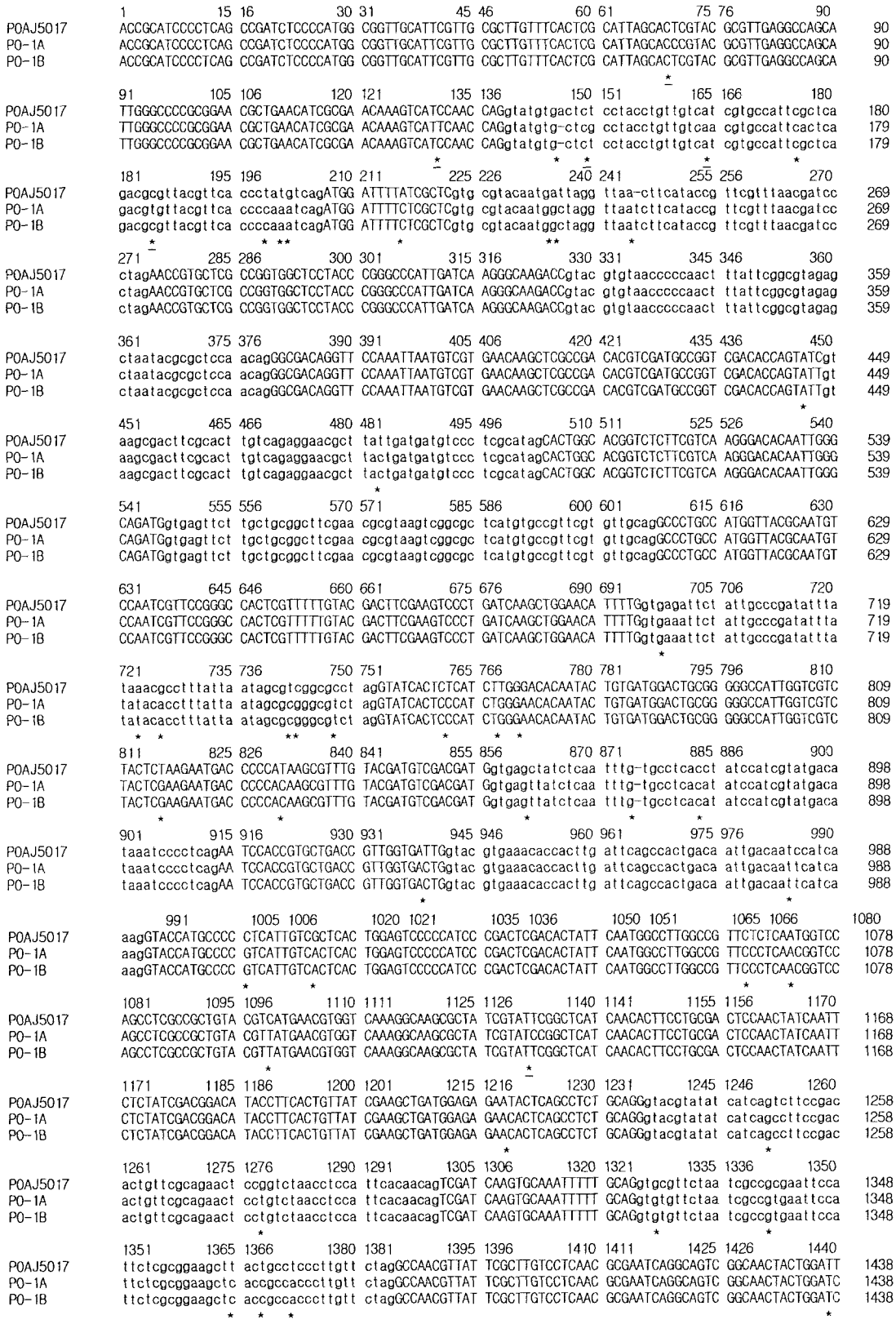

Fig. 1. Alignment of the *lac* genes of *Pleurotus ostreatus* Florida and PO-1. POAJ5017 is the *lac* gene of Florida. PO-1A is the *lac*-A gene and PO-1B is the *lac*-B gene of *P. ostreatus* PO-1. The different bases among three genes are shown by asterisks. The underlined asterisks are the different bases between *lac*-A and *lac*-B gene.

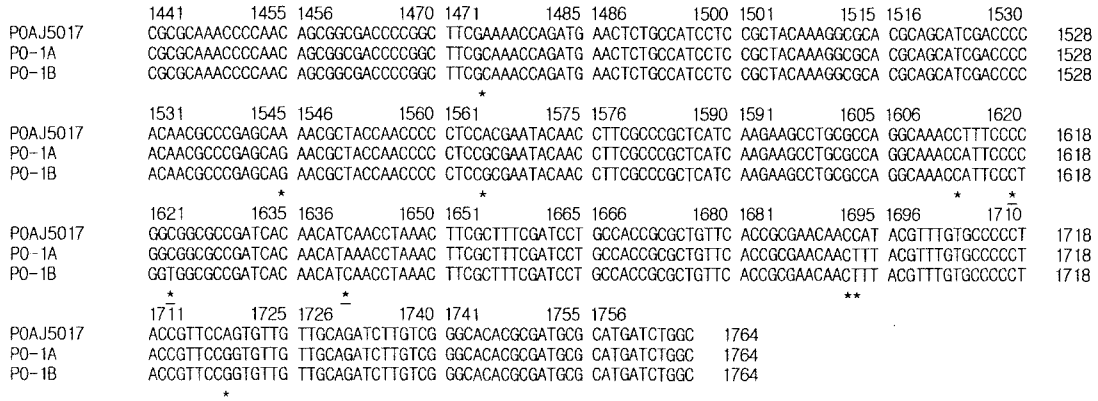

Fig. 1. Continued.

cloned into pGEM T-vector (pGEM T-easy Vector System, Promega). The cloned genes were sequenced by automatic sequencer (LI-COR IR2 System). The sequences were analyed with BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331).

RESULTS

1. Comparison of *lac* gene structures between *P. ostreatus* PO-1 and Florida strain

With LAC-A1 (from -26 nt to -7 nt above the ATG start codon of POAJ5017) and LAC-B1 (from 1717 nt to 1737 nt) primers, 1764 bp fragment of *lac* gene was amplified from PO-1. However, the expected product of PCR with LAC-A1 and LAC-B2 (contains the TGA terminal codon) was not amplified. Therefore, the 1764 bp fragment of *lac* gene was used for analysis. From the PCR products of PO-1, two types of *lac* genes were cloned as *lac*-A and *lac*-B. The *lac*-A gene differed 10 bases of 1764 bases from *lac*-B gene of PO-1 (Fig. 1). The sequences of the *lac*-A and *lac*-B differed from that of the *lac* gene (POAJ5017) of Florida strain as 54 and 48 bases, respectively (Fig. 1). Among 386 residues of the putative amino acid sequence of Lac-A, Pro-15 differed from Leu-15 in Lac-B of PO-1 and laccase of Florida strain (Fig. 2). Additionally four residues (Ser-41, Ala-290, Arg-320 and Phe-363) were simultaneously differed in Lac-A and -B from laccase of Florida strain (Tyr-41, Glu-290, His-320 and His-363, respectively).

2. Mutation spectra of *lac* genes induced by gamma ray radiation

All partial *lac*-A genes of 4 mutants (PO-6, -7, -14 and -15) consisted of 1763 base pairs due to the deletion of two bases (491-nt and 493-nt) and addition of one base (875-nt) of PO-1 (Table 1). Total 36 hotspots were detected in the partial *lac*-A gene of PO-1. Among them, 32 positions were simultaneously mutated in all of those 4 mutants, three positions were mutated in PO -6, -7 and -15, and one position was also mutated in PO-6, -14 and -15. Additionally PO-7 and PO-14 were mutated in 6 and 19 different positions, respectively. Twenty seven of the hot spots were concentrated between 70-nt and 884-nt of *lac*-A gene. Therefore, the mutation rates of *lac*-A gene induced by gamma radiation was 3.2% (58 bp/1764 bp). These mutations were grouped of predominantly transitions (77.1%) and followed by transversions (18.0%), base deletion (3.2%) and base insertion (1.6%) (Table 2). The specific mutated positions in *lac*-A of PO-14 ranged from 1015-nt to 1730-nt except one mutated base at 724-nt (Table 1). By the reirradiation of 1 kGy, all of these specific mutated bases of *lac*-A gene of PO-14 reversed to normal bases in the *lac*-A gene of PO-15, and additionally 3 hot spot positions of PO-15 were mutated at 903-nt, 985-nt and 1006-nt witch were same mutated bases in PO-6 and -7. However, back mutations of hot spots in PO-14 were not occurred by the reirradiation due to same mutations were found in the mutant PO-15.

The 1764 bp of partial *lac*-B gene was cloned only in PO-5 mutant and contained 20 mutated bases (the mu-

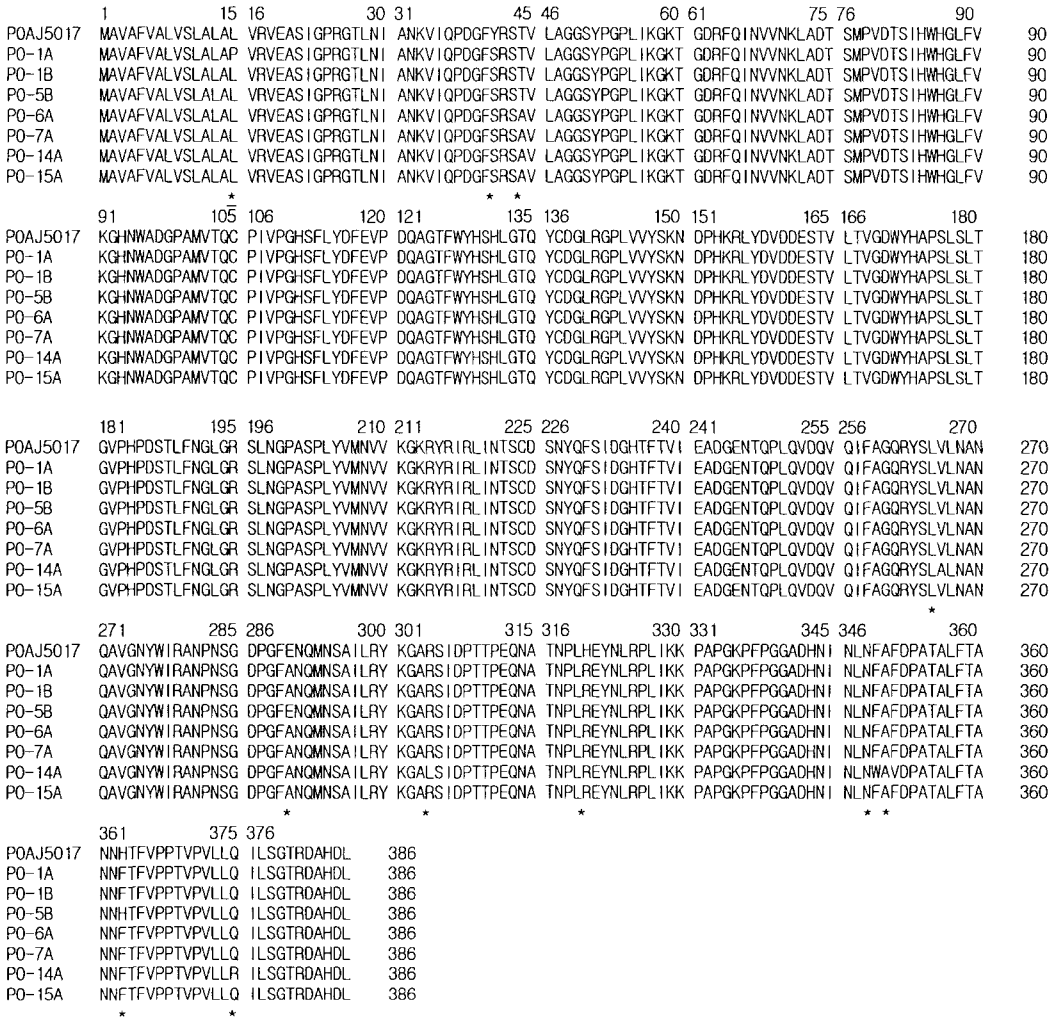

Fig. 2. Alignment of the amino acid sequences of the Lac proteins of *Pleurotus ostreatus* PO-1 mutants induced by gamma ray radiation. POAJ5017 is the Lac protein of *Pleurotus ostreatus* Florida. The Lac-A protein is indicated by "A" and the Lac-B protein is indicated by "B". The different amino acids among eight enzymes are shown by asterisks. The underlined asterisk is the different amino acid between Lac-A and Lac-B protein.

tation rate was 1.1%; Table 1). No deletion or insertion of base was found in *lac*-B gene of PO-5. The mutated bases were distributed between 1015-nt and 1719-nt and consisted of transition (75%) and transversion (25%) (Table 2). Surprisingly, 14 bases among 20 point mutations found in *lac*-B of PO-5 were equal to the specific mutated bases in *lac*-A of PO-14. It seemed that these positions may be candidates of the general hot spot sites between *lac*-A and *lac*-B genes of *P. ostreatus* induced by gamma ray radiation. The frame shift mutation was not found in *lac*-A and *lac*-B genes of all mutants.

3. Changes of the putative amino acid residues of *lac* genes in mutants

Two simultaneously changed residues (Pro-15Leu-15 and Thr-44Ala-44) were found in the putative amino acid sequences of *lac*-A genes of PO-6, -7, -14 and -15 mutants. Lac-A protein of PO-14 has additionally 5 changed residues as Val-266Ala-266, Arg-304Leu-304, Phe-349Trp-349, Phe-351Val-351 and Gln-375Arg-375. Lac-B protein of PO-5 has two changed residues as Ala-290Glu-290 and Phe-363His-36 (Table 1, Fig. 2).

Table 1. Sequence analysis of *lac* genes of gamma ray radiation-induced mutants in *Pleurotus ostreatus* PO-1

Position	Exon/ Intron	*lac*-A gene					*lac*-B gene		Amino acid changes	Amino acid number	Target sequence 5′ to 3′
		PO-1	PO-6	PO-7	PO-14	PO-15	PO-1	PO-5			
70	E1	C*	T	T	T	T			P → L	15	AGCAC C CGTAC
247	I2	t	c	c	c	c					taatc t tcata
268	I2	t	c	c	c	c					aacga t cccta
276	E3	A	G	G	G	G			T → A	44	ctagA A CCGTG
342	I3	a	g	g	g	g					ccccc a acttt
365	I3	t	c	c	c	c					gctaa t acgcg
370	I3	g	a	a	a	a					tacgc g ctcca
388	E4	G	A	A	A	A			R → R		GACAG G TTCCA
397	E4	T	C	C	C	C			I → I		CAAAT T AATGT
418	E4	C	T	T	T	T			A → A		CTCGC C GACAC
448	E4	T	C	C	C	C			I → I		AGTAT T gtCAG
465	I4	t	c	c	c	c					cgcac t tgtca
483	I4	c	t	t	t	t					gctta c tgatg
488	I4	g	a	a	a	a					ctgat g atgtc
491	I4	g	Deletion	Deletion	Deletion	Deletion					atgat g tccct
492	I4	t	Deletion	Deletion	Deletion	Deletion					tgatg t ccctc
530	E5	A	C	C	C	C			G → G		AAGGG A CACAA
663	E6	C	T	T	T	T			D → D		TACGA C TTCGA
700	I6	a	g	g	g	g					Ggtga a attct
724	I6	a			g						tatat a cacct
726	I6	a	g	g	g	g					tatac a ccttt
742	I6	c	t	t	t	t					tagcg c gggcg
743	I6	g	c	c	c	c					agcgc g ggcgt
762	E7	C	T	T	T	T			S → S		CACTC C CATCT
766	E7	C	T	T	T	T			L → L		CCCAT C TGGGA
789	E7	A		G					G → G		GATGG A CTGCG
859	I7	g	a	a	a	a					ATGgt g agtta
862	I7	t	c	c	c	c					gtgag t tatct
869	I7	a	g	g	g	g					atctc a atttg
871	I7	Gap	t	t	t	t					atttg - tgcct
884	I7	a	c	c	c	c					ctcac a tatcc
886	I7	a		g							cacat a tccat
903	I7	a	c	c		c					acata a atccc
936	E8	T		C					G → G		GTTGG T GACTG
985	I8	t	c	c		c					acaat t catca
1006	E9	G	C	C		C			P → P		GCCCC G TCATT
1015	E9	A			G		A	G	S → S		TTGTC A CTCAC
1036	E9	C			T		C	T	P → P		CATCC C GACTC
1069	E9	C			T		C	T	S → S		CGTTC C CTCAA
1099	E9	T			C		T	C	V → V		TACGT T ATGAA
1129	E9	T			C				R → R		TATCG T ATCCG
1132	E9	C	T	T	T	T			I → I		CGTAT C CGGCT
1219	E9	C			T		C	T	N → N		GAGAA C ACTCA
1239	I9						c	t			GGgta c gtata
1252	I9	c			t		c	t			atcag c cttcc
1278	I9	t			g		t	g			actcc t gtcta
1328	I10	t			c		t	c			AGgtg t gttct
1365	I10	c			t		c	t			aagct c accgc
1368	I10	c			t		c	t			ctcac c gccac
1395	E11	T		C					Y → Y		CGTTA T TCGCT
1401	E11	T		C					I → I		TCGCT T GTCCT
1403	E11	T			C				V → A	266	GCTTG T CCTCA
1440	E11	C			T		C	T	I → I		TGGAT C CGCGC
1475	E11						C	A	A → E	290	CTTCG C AAACC
1517	E11	G			T				R → L	304	CGCAC G CAGCA
1620	E11	C	T		T	T	T	C	P → P		TTCCC C/T GGCGG
1623	E11	C	T	T	T	T	T	C	G → G		CCCGG C/T GGCGC
1641	E11	A	C	C	C	C	C	A	I → I		AACAT A/C AACCT
1652	E11	T			G				F → W	349	AAACT T CGCTT
1653	E11	C			G				F → W	349	AACTT C GCTTT
1657	E11	T			G				F → V	351	TCGCT T TCGAT
1668	E11						C	G	A → A		CCTGC C ACCGC
1693	E11						T	C	F → H	363	ACAAC T TTACG
1694	E11						T	A	F → H	363	CAACT T TACGT
1713	E11	C		T					T → T		CCTAC C GTTCC
1719	E11						G	A	P → P		GTTCC G GTGTT
1730	E11	A							Q → R	375	GTTGC A GATCT

* A small letter is a base in intron and a capital letter is a base in exon. Blank is the same base of PO-1. Nucleotide numbered according to Fig. 1.

Table 2. Mutations induced by gamma ray radiation on *lac* genes of *Pleurotus ostreatus* PO-1

Mutation type	*lac*-A (1764 bp)	*lac*-B (1764 bp)
Transitions		
A→G	10/61 (16.4%)	1/20 (5.0%)
G→A	4/61 (6.6%)	1/20 (5.0%)
T→C	15/61 (24.6%)	5/20 (25.0%)
C→T	18/61 (29.5%)	8/20 (40.0%)
Transversions		
A→C	4/61 (6.6%)	–
G→T	1/61 (1.6%)	–
G→C	2/61 (3.3%)	–
C→A	–	2/20 (10.0%)
C→G	1/61 (1.6%)	1/20 (5.0%)
T→A	–	1/20 (5.0%)
T→G	3/61 (4.9%)	1/20 (10.0%)
Deletions*		
G→(−)	1/61 (1.6%)	–
T→(−)	1/61 (1.6%)	–
Insertions		
(−)→T	1/61 (1.6%)	–

*. These deletions occurred at tandem sequences as 5′-GT-3′.

DISCUSSION

In this study, the mutation spectra of *lac* genes were investigated in the mutants which induced by gamma ray radiation from edible mushroom, *P. ostreatus* PO-1. Previously, these mutants were independently isolated from the survivals of the mycelial fragments after irradiation of 1~2 kGy dose range of gamma ray radiation and confirmed the enhanced ability of ligninolysis and the diversity of genetic similarity by RAPD analysis (Lee *et al.* 2000). Therefore, it assumed that the DNA sequences of *lac* gene which is the main enzyme to degrade lignin may be mutated and that the mutation spectra of these genes include the common positions as the hot spot and the specific positions in individual mutants. These information were used to evaluate the tolerant limitation of change in the functional genes at least *lac* genes in this eukaryotic organism and to modify the molecular structure of proteins through changing the structural genes by gamma ray irradiation.

In this study we found two laccase genes as *lac*-A and *lac*-B which consisted of 10 different bases from *P. ostreatus* PO-1 (Fig. 1). We amplified the two genes by PCR method with same primer set. Unfortunately, we have not cloned both *lac* genes from same mutant. Therefore, it is not clear whether error of PCR or not. However, *P. ostreatus* PO-1 and it mutants have a dicaryotic mycelium, each cell of the dicaryon containing two nuclei, one derived from each mating type. It is suggests that two *lac* genes are an allele of laccase gene of *P. ostreatus*.

Most of the gamma ray induced mutations in our target genes were base substitutions (97%) (Table 1), a result which was also obtained by other studies (Miles and Meuth 1989; Sargentini and Smith 1994a; Wijker and Lafleur 1998; Wijker *et al.* 1996, 1998). The majority of all types of base substitutions occurred on G and C base in other studies (Sargentini and Smith 1994b; Wijker *et al.* 1996), whereas our result showed clear difference. In *lac*-A gene, the A and T bases were 52.5% of all base substitutions, but 45% in the *lac*-B gene. The predominance of mutations at C bases could partly be explained by the fact that the *lacI* gene had more C sites than A sites (Wijker *et al.* 1996). It may be true in case of *lac*-B because 53.37% of bases consisted of C and G bases, and the base substitution of C and G bases was 60%. Although the contents of C and G bases of *lac*-A (53.33%) were higher than A and T bases, A and T bases were more mutated in *lac*-A gene. Since the mutation spectrum could be resulted from the repair of the damaged base or base pair by the DNA repair system of cell, the damaged bases induced by gamma ray radiation could have no preference for a specific base (or base pairs) as suggested by Sonntag (1987). But the mutation spectrum could reflect on a specific DNA sequence or kinds of certain genes dependent on the repair mechanisms of cell. This is supported by the fact that the 41 bases of same positions in *lac*-A of PO-6 and PO-15 mutants were mutated, but 20 bases were mutated in *lac*-B of PO-5 mutant, though the sequence of *lac*-A differed only 10 bases of 1764 bases from *lac*-B gene. Additionally only three positions of the 10 different positions between *lac*-A and *lac*-B were commonly mutated (Table 1).

In gamma ray radiation induced mutation spectra described here, the deletion only accounted for 1 type as 2 tandem-arrayed bases at 491-nt and 492-nt in *lac*-A gene (Table 1). Studies on ionizing radiation induced mutagenesis in mammalian cell lines have shown that large deletions are the main type of induced mutations

(Thacker 1986; Miles and Meuth 1989; Nelson *et al.* 1994; Thacker 1999). In *E. coli*, the size of the deletions were from one base to several hundred bases (Wijker *et al.*, 1996). This difference in the amount of induced deletions could be due to differences in repair of double strand breaks in prokaryotic and eukaryotic cells. Because the mutants used in this study were isolated with several criteria such as growth rate in lignin medium and formation of fruiting body, the mutants with more severally deleted mutations could be excluded. Four mutants studied on *lac*-A gene mutation spectrum have also one base insertion at same positions. These deletion and insertion mutations occurred in intron regions. Therefore, frame shift mutations did not occur in this gene.

It seemed that PO-1 strain of *P. ostreatus* (isolation from Korea) has different *lac* gene from other strain, Florida (*P. ostreatus* (Jacq.: Fr.) Kummer, type, Florida) (Giardina *et al.*, 1995) (Fig. 1). These strains may evolve individually into other strains through genetic barrier of regional separation. Therefore, they would have different spectrum of *lac* gene naturally by spontaneous mutation (Fig. 1). The mutational hot spots which mutated to same base at same position of all studied mutants were found in *lac*-A gene as 55.17% of all base substitutions (Table 1). The *lac*-A gene of PO-1 strain consisted of 32 hot spots against gamma ray radiation. Among them, 14 sites were equal to the natural mutated sites and were mutated by gamma ray radiation to same bases as those of Florida strains (Fig. 1, Table 1). Additionally 10 mutated sites found in *lac*-A gene of PO-7 mutant were also mutated to same bases as those of Florida strain. Due to *lac*-A gene of PO-1 differed 58 bases from that of Florida strain by natural mutation, the hot spots were ranged from 24% to 41% of natural mutation sites. Lac-B gene was only cloned from PO-5 mutant and it was impossible to find out the hot spot sites, but the 16 sites of 20 mutated bases induced by gamma ray radiation were mutated at same bases to those of Florida strain and consisted of 33% of the natural mutations (Fig. 1, Table 1). This phenomenon has not been detected previously in other studies. Most of the hot spots of *lac* genes of PO-1 determined in this study could not be mutated backward to original or other bases by the reirradiation of gamma ray radiation (Table 1). It seems likely that the sites of hot spots are changed by gamma ray radiation to certain base as found in mutants of PO-1 and then altered the property of muta-tional hot spots. Although the mutated bases induced by gamma ray radiation of the hot spots in PO-1 were not changed to other bases by reirradiation, these hot spots could be changed by other dose of gamma ray radiation or the irradiated frequency due to half of the mutated bases were same to that of the other strain as Florida (Fig. 1). In the *lacI* gene model system in *E. coli* after in vivo and in vitro irradiation, the clear hot spots have not been induced by gamma ray radiation, although some clear hot spots were spontaneously induced (Nelson *et al.* 1994; Wijker *et al.* 1996).

The *lac* genes of PO-1 mutants produce the different proteins with the different amino acids (Fig. 2). In this study the properties of the mutated proteins have not determined, but these products of *lac* genes of mutants could be useful for degrading the recalcitrant lignin of biowastes due to these mutants showed an enhanced ligninolytic activity as previously mentioned (Lee *et al.* 2000). The DNA fragments of *lac* genes of mutants could also be a useful material for genetic engineering. From this results it is suggested that gamma ray radiation could induce the simultaneous mutation spectrum between mutants with some specific mutated bases in same genes of edible mushroom, *P. ostreatus*.

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