Enzymatic and structural studies of glutathione S-transferases of white-rot fungus *Ceriporiopsis subvermispora* which is a selective degrader of lignin in woody biomass

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The woody biomass has three major components; cellulose, hemicellulose and lignin. Lignin, one of the wood components, is becoming important as a raw material as to sustainable biorefinery. Since lignin is composed of phenylpropanoid units, the oxidative breakdown of lignin by extracellular enzymes could potentially release low molecular weight recalcitrant organic compounds. White-rot fungi are organisms that mineralize all components of the plant cell wall and possess intracellular networks to deal with these compounds. The intracellular detoxification system comprises three phases, namely, oxidation by P450, GSH-conjugation by glutathione S-transferases (GSTs), and excretion. GSTs are multifunctional enzymes and capable of catalyzing detoxification reactions and are also involved in endogenous metabolism. In this study, we focused on the two GSTFuA class GSTs of white-rot fungus *C. subvermispora* (CsGSTs), namely CsGST63524 and CsGST83044.

In chapter 1, firstly, a general introduction regarding the woody biomass and white-rot fungi was given. Then, the general introduction on the extracellular and intracellular enzymes of white-rot fungi were also provided. Finally, the background, functions and structures of the GSTs were described.

In chapter 2, the development of the expression of two CsGSTs in *Escherichia coli* was described. The genes of two CsGSTs were selected, codon-optimized and expressed. After two-step purification, as much as 150 mg of purified CsGSTs was obtained from 1L culture. The purified CsGSTs were further enzymatically characterized using different activity assays. The findings suggest that CsGSTs are involved not only in glutathionylation but also in the antioxidative process in cells. CsGST83044 exhibited etherase activity toward one of the lignin-like fluorogenic substrates, MUAV. CsGST63524 exhibited the highest esterase activity toward CMFDA among the studied GSTs. The analyses of the etherase and esterase activities revealed that the two CsGSTs exhibit different substrate specificities, which implies that each CsGST may play a distinct role in the metabolism of wood-derived compounds.

In chapter 3, the structures of CsGST63524 in GSH-free and -bound forms were analyzed by X-ray crystallography. A functional mutagenesis study was carried out to identify the important residues involved in the enzymatic activities of CsGST63524. In this study, CsGST63524 was purified using three steps of purification comprising affinity, anion exchange, and size exclusion column chromatography in order to improve the purity for crystallization purposes. The structure of CsGST63524 in a complex with GSH revealed that the sulfur atom of GSH forms a hydrogen bond with Ser21 of CsGST63524, indicating it is a serine-type GST. The functional mutagenesis of Ser21 unexpectedly indicated that this serine residue is not essential for the enzymatic activity of CsGST63524. Comparative sequence and structural analyses, together with functional mutagenesis, newly identified the enzymatically important non-canonical amino acid residues, Asn23 and Tyr45, other than the serine residue. The putative substrate-binding site of CsGST63524 was deduced based on structure-based sequence alignment and structural comparison with PcGSTFuA1 and LigE. Trp20, Pro22, and Trp217 of CsGST63524 might be involved in the substrate recognition. Tyr45, Ser120, Val124, Arg125, Ala128, Pro129, Leu132, Ser154, Ala155, Trp163, and Trp214 may also be components of the substrate-binding site of CsGST63524.

In chapter 4, the structures of CsGST83044 in GSH-free and -bound forms were analyzed by X-ray crystallography. A functional mutagenesis study was carried out to identify the key residues for the various enzymatic activities of CsGST83044. The structure of CsGST83044 in a complex with GSH revealed the residues that are involved in direct hydrogen bonding with GSH, Asn22, Asn24, Lys27, Tyr46, Leu75, Ser90, Tyr150, and Arg155. Asn22, Asn24, and Tyr46 are the residues located closest to the sulfur atom of GSH. Structure-based sequence alignment in combination with structural comparison of CsGST83044 with other GSTs indicated that CsGST83044 is not either a serine-, tyrosine-, or cysteine-type GST, but an atypical-type. Functional mutagenesis indicated that Asn22, Asn24, and Tyr46 are crucial for the enzymatic activity of CsGST83044. The Y46A mutant showed decreased k_{cat} values for the enzymatic reactions on all substrates, which indicated that this tyrosine residue is very important for the enzymatic activities of CsGST83044. The N22A and N24A mutants also exhibited decreased k_{cat} values for the enzymatic reactions on most of the substrates, which indicated that these two asparagine residues are important for the enzymatic activities of CsGST83044 as well. The hydrogen bonding network among Asn22, Asn24, and Tyr46 in CsGST83044 may help to stabilize the interaction between these residues with the sulfur atom of GSH. The putative substrate-binding site of CsGST83044 was also deduced on the basis of the structure-based

sequence alignment and structural comparison with CsGST63524. All of the residues comprising the putative substrate-binding site are located close to the GSH-bound pocket. The size of the GSH-bound pocket of CsGST83044 turned out to be smaller than that of CsGST63524, which rationally explains the substrate preferences of these GSTs. The previous study indicated that CsGST83044 exhibits higher esterase activity toward 4MUA, which is less bulky than CMFDA. It is assumed that a less bulky substrate can be properly located in the small pocket of CsGST83044.

In chapter 5, a general conclusion was given.

In this study, we have characterized the two GSTFuA class GSTs of *C. subvermispora*, a selective degrader of lignin in woody biomass. Our findings showed that CsGST63524 exhibit the highest esterase activity among the studied GSTs. CsGST83044 exhibits etherase activity for the first time for fungal GSTs. To date, all GSTFuA class GSTs have a serine residue that located closest to the sulfur atom of GSH, and known as serine-type GSTs. CsGST63524 has Ser21 that forms a hydrogen bond with the sulfur atom of GSH. However, the functional mutagenesis study revealed that serine is not crucial for the enzymatic activity. In this study, we found the amino acid residues other than serine one that can play a key role in enzymatic activities of the GSTFuA class. Asn23 and Tyr45 of CsGST63524 are next located closest to the sulfur atom of GSH. The functional mutagenesis indicated that Asn23 and Tyr45 are crucial for the enzymatic activity of CsGST63524. This is the first identification of amino acid residues other than the serine residue that can play a key role in enzymatic activity of serine-type GSTs. We have categorized CsGST83044 as an atypical-type GST belonging to a GSTFuA class based on the structure-based sequence alignment in combination with structural comparison with other GSTs. CsGST83044 has three residues which are Asn22, Asn24, and Tyr46 that form hydrogen bonds with sulfur atom of GSH. The functional mutagenesis study revealed that all these there residues are important for the CsGST83044 enzymatic activities. This is the first identification of the critical residues for the enzymatic activities of an atypical-type GST belonging to GSTFuA class. Altogether, the asparagine and tyrosine are important for the enzymatic activity of CsGSTs that belong to the GSTFuA class. We have also provided information on the potential hydrophobic residues that might be involved in the substrate preference of both enzymes based on structural comparison with other well-known GSTs. The engineering of the GSTs of *C. subvermispora* will be useful in biotechnology such as for the utilization of lignin in the woody biomass.