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Site-directed Mutagenesis of Cysteine Residues in Phi-class Glutathione *S-transferase* F3 from *Oryza sativa*

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To elucidate the roles of cysteine residues in rice Phi-class GST F3, in this study, all three cysteine residues were replaced with alanine by site-directed mutagenesis in order to obtain mutants C22A, C73A and C77A. Three mutant enzymes were expressed in *Escherichia coli* and purified to electrophoretic homogeneity by affinity chromatography on immobilized GSH. The substitutions of Cys73 and Cys77 residues in *Os*GSTF3 with alanine did not affect the glutathione conjugation activities, showing non-essentiality of these residues. On the other hand, the substitution of Cys22 residue with alanine resulted in approximately a 60% loss of specific activity toward ethacrynic acid. Moreover, the K_m^{CDNB} value of the mutant C22A was approximately 2.2 fold larger than that of the wild type. From these results, the evolutionally conserved cysteine 22 residue seems to participate rather in the structural stability of the active site in *Os*GSTF3 by stabilizing the electrophilic substrates-binding site's conformation than in the substrate binding directly.

Key Words : Cysteine residues, Rice, Glutathione *S*-transferase, Substrate specificity, Kinetic parameters

Introduction

Glutathione *S*-transferase (GST, EC 2.5.1.18) is a family of multifunctional proteins, catalyzing the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds. In plants, GST plays a pivotal role in the detoxification of herbicides, pesticides, organic pollutants and natural toxins.[1,2] Certain GSTs also catalyze peroxidase reactions or isomerization of certain steroids and are involved in hydroxyl peroxidase detoxification or tyrosine metabolism, respectively.[3-5] Other GSTs play an important role in the intracellular transport of numerous nonsubstrate ligands such as auxins and cytokinins[3,4,6] or anthocyanins,[7,8] and thus contribute to hormone homeostasis or vacuolar anthocyanin sequestration, respectively.

GSTs are distributed in a wide range of organisms from mammals to *E. coli*.[9] Plant GSTs have been concerned in agricultural chemistry and biochemistry because they are one of the major factors involved in the resistance of a variety of herbicides.[10] Based on sequence similarity and exon structure, plant GSTs have been subdivided in class phi (F), zeta (Z), tau (U), theta (T) and lambda (L).[3,11,12] Individual GST isozymes can selectively detoxify specific xenobiotics with species differences in GST specificity and capacity determining herbicide selectivity.[4,11,13] In plants, studies regarding GSTs have focused mainly on their ability to detoxify herbicides.

Many GSTs from plants have been purified and their genes have been cloned. Among them, the enzymes from *Arabidopsis*, soybean, and maize have been extensively studied in terms of their structure, function, and physiological significance.[3,14] However, the structure and functions of GST subunits from rice, an important food in Asia, are poorly understood.[15-21] Moreover, there is little information available concerning the precise enzyme-substrate interactions that may be responsible for the catalytic properties of rice GST and the identification or specific role of individual residues remains largely unknown.

All three cysteine residues are present in *Os*GSTF3. Among them, Cys22 is only conserved in all the known phi class GSTs (Fig. 1). In this study, three cysteine residues (at position 22, 73 and 77) in *Os*GSTF3 were replaced with alanine by oligonucleotide-directed mutagenesis and the effect of the replacements on the enzymatic activity were examined in order to evaluate the role of these residues. This study offers information on the precise enzyme-substrate interactions responsible for the catalytic properties of *Os*GSTF3, and it will be of great value in designing new rice-specific herbicides and pesticides.

Experimental Section

Materials. GSH and 1,2-dichloro-4-nitrobenzene (CDNB) were purchased from Kohjin Co. and Wako Pure Chem. Ind. (Osaka, Japan), respectively. Cumene hydroperoxide (CP), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), ethacrynic acid (ETA) and GSH-agarose were obtained from Sigma (St. Louis, USA). All other reagents used were of the highest grade commercially available.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CP, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; ETA, ethacrynic acid; GSH, glutathione; G-site, glutathione-binding site; GST, glutathione *S*-transferase; *Os*GST F3, rice Phi-class GST F3; H-site, electrophilic substrate-binding site; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

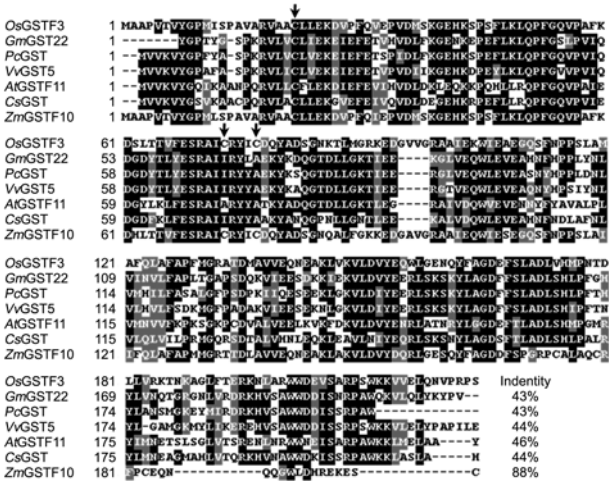

Figure 1. Comparison of amino acid sequence of the *Os*GST F3 and other phi class GSTs. The sequences have been aligned with dashes indicating gaps. The regions conserved are shaded in black. The arrow indicates the amino acids that mutated cysteine residues in the present study. This sequence alignment was created using the following sequences; (Organism, NCBI protein ID) *Os*GST F3 (*Oryza sativa*, AAG32477); *Gm*GST22 (*Glycine max*, AAF34812); *Pc*GST (*Pyrus communis*, ABI79308); *Vv*GST5 (*Vitis vinfera*, ABW34390); *At*GSTF11 (*Arabidopsis taliana*, NP186969); *Cs*GST (*Citrus sinensis*, ABA42223); *Zm*GSTF10 (*Zea may*, AAG34818).

Preparation of Mutant Enzymes by Site-directed Mutagenesis. Wild-type *Os*GSTF3 was obtained by expression of a cloned cDNA in *E. coli* as described in the previous paper.[16] The oligonucleotide primers used for site-directed mutagenesis of Cys22, Cys73 and Cys77 into Ala are shown in Table 1. Mutagenesis was performed according to the Mutant^{TM}-Super Express Km Kit protocol (Takara Shuzo). Construction of the DNA template for mutagenesis, confirmation of mutation and construction of the expression plasmid in regards to the mutants were performed as described in a previous study.[22] The resulting vectors of the mutant proteins were transformed into *E. coli* strain BL21 Star (DE3).

Overexpression and Purification of Mutant Enzymes. The mutant enzymes were expressed in *E. coli* under the control of the *tac* promoter. Cultured cells were lysed, followed by centrifugation. The dialyzed supernatant of the cell lysate was loaded directly onto a 15 mL column of GSH-agarose equilibrated with a 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The column was extensively washed with the same buffer. The enzyme was eluted with a 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM GSH and dialyzed against buffer A. This dialyzed purified enzyme was used for the next experiment. Unless otherwise indicated, all purification procedures were carried out either at 4 °C or on ice.

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutant	Sequence of primer[a]	Sense
C22A	5'-GTGGCGGCC<u>GC</u>CGTGCTGGAG-3'	Forward
C73A	5'-AGGGCTATT<u>GCC</u>CGTTACATC-3'	Forward
C77A	5'-CGTTACATC<u>GCT</u>GACCAGTAC-3'	Forward

[a]Changed bases are shown by underlines.

Protein Assay and Electrophoresis. Protein concentration of the wild-type and mutant enzymes was determined by using a protein assay reagent (Bio-Rad Lab.) as described in the previous paper.[21] Denaturing SDS-PAGE was carried out in 12.5% gels with SDS molecular weight standard markers (Bio-Rad, USA). The gels were then stained with Coomassie Blue R-250.

Enzyme Activity. The specific activities were determined by measuring the initial rates of the enzyme-catalyzed conjugation of GSH with CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), EPNP, 4-nitrophenethyl bromide (NPB) and (ETA).[23] An unit of activity is defined as the amount of enzymes catalyzing the formation of 1 μmole of product per min under the conditions of the specific assay. Specific activity is defined as the units of enzyme activity per mg of protein. GSH-dependent peroxidase activity was assayed as described by Flohe and Güzler.[24]

Kinetic Studies. Kinetic studies with GSH and CDNB were carried out at 30 °C as described in the previous paper.[16] The kinetic parameter K_m values were determined under first order conditions at a low substrate concentration with respect to the varied substrate: for GSH with a fixed concentration of 1 mM CDNB, and for CDNB with a fixed concentration of 2.5 mM GSH. The k_{cat} values were calculated on the basis of the mol dimeric enzyme using a M_r of 48,000. Other experimental conditions were the same in regards to the determination of specific activities.

Results

Purification of Mutant Enzymes. To elucidate the role of cysteine residues in *Os*GSTF3, we substituted cysteine residues (at position 22, 73 and 77) with alanine using oligonucleotide-directed mutagenesis. The mutant enzymes were expressed in *E. coli* under the control of a *tac* promoter. The expressed mutant enzymes were isolated and purified by affinity chromatography on immobilized GSH. The C22A, C73A and C77A mutants were isolated in a yield of approximately 4-8 mg per liter of cultures as in the case of the wild-type enzyme, showing that the binding abilities of mutant enzymes to GSH-agarose were not much different from that of the wild-type enzyme. The purified mutant enzymes give a single band on the SDS-PAGE with an apparent *M*r of 24 kDa equivalent to that of the wild-type enzyme (Fig. 2).

Effects of Point Mutations of Cysteine Residues on Substrate Specificity. The substrate specificities of the mutant enzymes are shown in Table 2. The *Os*GSTF3 and the mutant enzymes showed no activities towards DCNB for mu class GST, NPB for theta class GST, and CP for glutathione peroxidase activity. The substitutions of Cys22, Cys73 and Cys77 with alanine had a negligible effect on the GSH conjugation activity towards CDNB. On the other hands, the substitutions of Cys73 and Cys77 with alanine resulted in

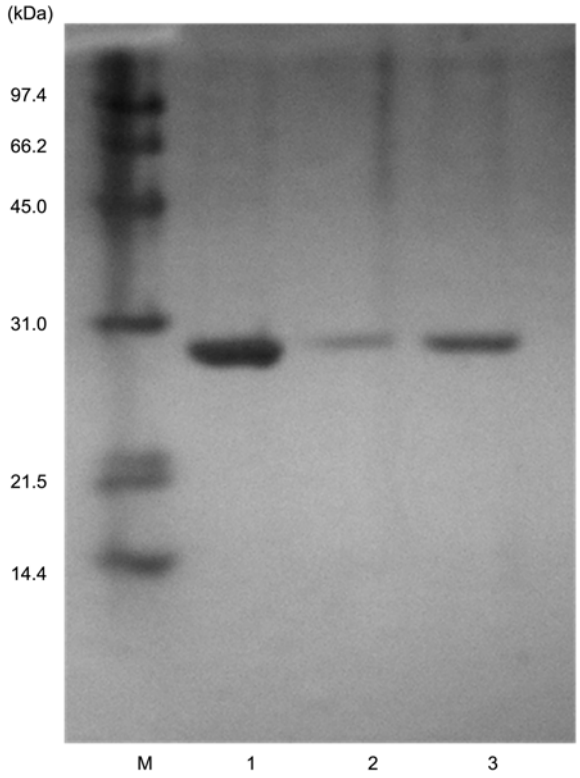

Figure 2. SDS-PAGE of mutant enzymes. Lane M, SDS-low range molecular weight marker; lane 1, purified C22A; lane 2, purified C73A; lane 3, purified C77A.

Table 2. Substrate specificity of the *Os*GST F3 and mutant enzymes

Substrate	Specific activity (μmol/min/mg)			
	*Os*GSTF3	C22A	C73A	C77A
CDNB	7.60 ± 0.08	7.85 ± 0.09	7.23 ± 0.07	8.37 ± 0.13
CP	ND[a]	ND	ND	ND
DCNB	ND	ND	ND	ND
EPNP	0.48 ± 0.15	0.54 ± 0.14	0.32 ± 0.06	0.41 ± 0.13
ETA	0.47 ± 0.02	0.19 ± 0.01	0.42 ± 0.02	0.30 ± 0.02
NPB	ND	ND	ND	ND

Values are Means ± S.D., generally based on n ≥ 5. [a]ND - No detected.

approximately a 33 and 40% decrease of the specific activity towards EPNP and ETA, respectively. Particularly, the substitution of Cys22 with alanine showed a significant decrease (approximately 60%) in the specific activity towards ETA. These results demonstrate that the substitutions of the cysteine residues with alanine resulted in significant structural change regarding the electrophilic substrate binding site (H-site).

Effects of Point Mutations of the Cysteine Residues on Kinetic Parameters for GSH Conjugation Activity. The catalytic mechanism of CDNB conjugation has been the subject of many studies, since this reaction is the most commonly used assay for GST activity. Table 3 summarizes the kinetic parameters of the mutants for the GSH-CDNB conjugation. The substitution Cys22 with alanine resulted in approximately a 2.2-fold increase of K_m^{CDNB}, whereas the K_m^{GSH} and k_{cat}/K_m was similar to that of the wild type. These results suggest that the Cys22 in *Os*GSTF3 are not essential for the catalytic activity, but may contribute to some extent to the H-site. On the other hand, the substitutions of Cys73 and Cys77 with alanine scarcely affected the kinetic parameters.

Discussion

Plant-specific phi GSTs are more abundant compared to other classes and play important roles in stress tolerance and secondary metabolism as well as catalyzing the detoxification of herbicides in crops and weeds.[21,25] However, the relationship between structure and function of the phi class GST subunits from rice are still poorly understood. Major points of the catalytic mechanism in GST are the activation of the thiol group of reduced GSH for the nucleophilic reaction by deprotonation and the stabilization of the transition state for reactions with the electrophilic substrate. These results have mainly been obtained from studies on the relationship between the structure and functions of mammalian GSTs. Chemical modification studies have suggested that cysteine residues are located in the active site of GSTs.[26,27] The three-dimensional structures of GST also suggested the possibility that cysteine residues play an essential role in substrate binding affinity or catalysis.[28,29]

In the present study, in order to elucidate the precise enzyme-substrate interactions responsible for the catalytic properties of plant GSTs, we introduced site-directed mutations into three cysteine residues of the *Os*GSTF3 and examined the enzymatic properties of the mutated enzymes. It was found that the substitutions of Cys22, Cys73 and Cys77 with alanine had a negligible effect on the GSH conjugation activity (Table 2). From these results, we suggest that three cysteine residues of the *Os*GSTF3 are not the essential active-site residues. However, the substitution of an evolu-

Table 3. Kinetic parameters of the *Os*GST F3 and mutant enzymes for GSH-[1-chloro-2,4-dinitrobenzene] conjugation (CDNB)

Enzyme	GSH			1-Chloro-2,4-dinitrobenzene		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}S^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}S^{-1}$)
Wild type	0.25 ± 0.08	4.01 ± 0.21	16.0	0.85 ± 0.07	5.48 ± 0.21	6.5
C22A	0.23 ± 0.03	3.08 ± 0.08	13.4	1.88 ± 0.21	13.50 ± 1.06	7.2
C73A	0.18 ± 0.05	3.68 ± 0.25	20.4	1.24 ± 0.13	3.99 ± 0.32	3.2
C77A	0.22 ± 0.02	4.79 ± 0.36	21.8	0.81 ± 0.09	6.52 ± 0.18	8.1

Values are means ± SD, generally based on n ≥ 3.

tionarily conserved Cys22 with alanine significantly decreased the catalytic activity towards ETA (Fig. 1 and Table 2). Moreover, the substitution of Cys22 with alanine significantly affected K_m^{CDNB}, whereas it scarcely affected the binding ability to GSH-agarose, K_m^{GSH} and k_{cat} (Table 3). The K_m^{CDNB} of C22A was approximately 2.2-fold larger than that of the wild type. These results suggest that the substitution of Cys22 with alanine generally changes the binding affinity of the electrophilic substrate to the enzyme. The three-dimensional structures of phi class GSTs from *Arabidopsis* and maize suggested that the electrophilic substrate-binding sites had a broader and deep H-site when compared to the mammalian isoforms.[30,31] Neuefeind *et al.* have also reported that maize *Zm*GSTF1 binds atrazine in a number of different conformations with contributions from residues in both the *N*-terminal domain and helix α4.[32] Through preliminary data (studies are currently in progress) on the three-dimensional structure of *Os*GSTF3 in complex with GSH, Cys22 is not located in the G-site but apart from the catalytic site. Thus, this residue is expected not to participate directly in the catalytic mechanism. Taken together, the substitution of Cys22 is thought to change the binding affinity of the electrophilic substrate by inducing a conformational change of the H-site. This finding is evidence regarding the importance of structural residues that affect the enzymatic properties such as kinetic parameters and substrate specificity. Wongtrakul *et al*. have also reported a similar result from the site-directed mutagenesis of cysteine 69 residue in *AD*GSTD3-3 from *Anopheles dirus*.[33] From this study, we offered the in depth understanding concerning the detoxification mechanism of *Os*GSTF3 and the basic information for the development of herbicides acquiring high selectivity to a weed.

In conclusion, the presented results suggest that the evolutionally conserved cysteine 22 residue in *Os*GSTF3 seems to participate rather in the structural stability of the active site in *Os*GSTF3 by stabilizing the electrophilic substrates-binding site's conformation than in the substrate binding directly. On the other hand, Cys73 and Cys77 residues are not essential for the catalytic activity. Further clarification on the relationship between the structure and catalytic activity of these residues requires three-dimensional structure analysis with regards to these mutant enzymes.

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