http://dx.doi.org/10.5012/bkcs.2013.34.9.2671

Site-directed Mutagenesis of the Evolutionarily Conserved Tyr8 Residue in Rice Phi-class Glutathione *S*-transferase F3

Hyun-Joo Jo, Mi-Jin Pack, Jin-Young Seo, Jin-Kyung Lim, and Kwang-Hoon Kong*

*Biomolecular Chemistry Laboratory, Department of Chemistry, College of Sciences, Chung-Ang University, Dongjak-ku, Seoul 156-756, Korea. *E-mail: khkong@cau.ac.kr*
Received May 27, 2013, Accepted June 17, 2013

To elucidate the role of the evolutionarily conserved Tyr8 residue in rice Phi-class GSTF3, this amino acid was replaced with alanine and phenylalanine by site-directed mutagenesis, respectively. The replacement of Tyr8 with Ala significantly affected the catalytic activity and the kinetic parameters, whereas the substitutions of Tyr8 with Phe had almost no effect. The Y8A mutant resulted in approximately 90-100% decrease of the specific activity. Moreover, the Y8A mutant resulted approximately in 2-fold increase of K_m, approximately 60-80% decrease of k_{cat}, and approximately 6.5-fold decrease in k_{cat}/K_m. From the pH/log k_{cat}/K_m plot, pK_a values of the GSH in the wild-type enzyme-GSH complex, Y8A-GSH complex and Y8F-GSH complex were estimated to be approximately 6.8, 8.5 and 6.9, respectively. From these results, we suggest that the evolutionarily conserved Tyr8 residue in *Os*GSTF3 seems to influence the structural stability of the active site of *Os*GSTF3 rather than directly its catalytic activity.

Key Words : Tyrosine 8 residue, Rice, Glutathione *S*-transferase, Site-directed mutagenesis, Kinetic parameters

Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional enzymes involved in the metabolism of xenobiotics and reactive endogenous compounds. They catalyze the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds.[1,2] The interest in plant GSTs may be attributed to their agronomic value, since it has been demonstrated that glutathione conjugation for a variety of herbicides is the major resistance and selectivity factor in plants.[3] Based on sequence similarity and exon structure, plant GSTs have been subdivided in class Phi, Zeta, Tau, Theta and Lambda.[4-6] Individual GST isozymes can selectively detoxify specific xenobiotics with species differences in GST specificity and capacity determining herbicide selectivity.[5,7,8] In plants, studies regarding GSTs have mainly focused on their ability to detoxify herbicides.

The active sites of GSTs are suggested to be different in the location of a GSH-binding site (G-site) and a nonspecific hydrophobic site (H-site) to accommodate electrophilic substrates.[2] The catalytic mechanism of GSTs has been the target of many investigations involving kinetic and structural studies, chemical modification, site-directed mutagenesis and X-ray crystallographic analysis. In the Alpha, Mu, Pi, and Sigma classes of the human cytosolic GSTs, the primary active site residue is a tyrosine located in the N-terminal domain.[9] This tyrosine residue has been replaced by a serine residue in the Theta and Zeta classes and by a cysteine residue in the Omega class GSTs. However, the structure and functions of GST subunits from rice, an important food in Asia, are poorly understood. Moreover, there is little information 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

GSTs															
								⬇							
*Os*GSTF3	M	A	A	P	V	T	V	Y	G	P	M	I	S	P	A
*Gm*GST22	-	-	-	-	-	-	-	Y	G	P	T	Y	G	-	S
*Pc*GST	-	-	M	V	V	K	V	Y	G	P	F	Y	A	-	S
*Vv*GST5	-	-	M	V	V	K	V	Y	G	P	A	F	A	-	S
*At*GSTF11	-	-	M	V	V	K	V	Y	G	Q	I	K	A	A	N
*Cs*GST	-	-	M	V	V	K	V	Y	G	S	V	J	A	A	C
*Zm*GSTF10	M	A	A	P	V	T	V	Y	G	P	M	L	S	P	A

Figure 1. Comparison of the *N*-terminal amino acid sequences of the *Os*GSTF3 and other phi-class GSTs. The sequences have been aligned with dashes indicating gaps. The arrow indicates the evolutionally conserved Tyr8 residue that was mutated in the present study. This sequence alignment was created using the following sequences (Organism, NCBI protein ID): *Os*GSTF3 (*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).

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*GSTF3, rice Phi-class GSTF3; H-site, electrophilic substrate-binding site; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

residues.

There is a tyrosine located in the N-terminal domain of *Os*GSTF3. This Tyr8 residue is conserved in all the known Phi-class GSTs (Fig. 1). Highly conserved residues are expected to be important for the structure and/or function of enzymes. In this study, the Tyr8 residue in *Os*GSTF3 was replaced with Ala and Phe by oligonucleotide-directed mutagenesis and the effect of the replacements on the enzymatic activity were examined in order to evaluate the role of this residue.

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 were of the highest grade commercially available.

Preparation of Mutant Enzymes by Site-directed Mutagenesis. Wild-type *Os*GSTF3 was obtained by expression of a cloned cDNA in *Escherichia coli* as described in a previous study.[10] The oligonucleotide primers used for the site-directed mutagenesis of Tyr8 into Ala and Phe are shown in Table 1. Mutagenesis was performed according to the Mutant^{TM}-Super Express Km Kit protocol (Takara Shuzo, Shiga, Japan). Construction of the DNA template for mutagenesis, confirmation of mutation and construction of the expression plasmids were performed as described in a previous study.[11] 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 and centrifuged. The dialyzed supernatant of the cell lysate was loaded directly onto a 15-mL column of GSH-agarose equilibrated with 20 mM potassium phosphate buffer (pH 7.0) (buffer A). The column was extensively washed with the same buffer. The enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM GSH and dialyzed against buffer A. The 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.

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 a previous paper.[12] Denaturing SDS-PAGE was carried out in 12.5% gels with SDS molecular weight standard marker (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.[13] The activity of 1 unit of enzyme is defined as the amount of enzyme catalyzing the formation of 1 μmole of product per min under the conditions of the specific assay. Specific activity is defined as the unit of enzyme activity per mg of protein. GSH-dependent peroxidase activity was assayed as described by Flohe and Güzler.[14]

Kinetic Studies. Kinetic studies with GSH and CDNB were carried out at 30 °C as described in a previous study.[10] The values of the kinetic parameter K_m were determined under first order conditions at a low substrate concentration with respect to the varying substrate: GSH with a fixed concentration of 1 mM CDNB, and CDNB with a fixed concentration of 2.5 mM GSH. The k_{cat} values were calculated on the basis of the moles of dimeric enzyme using a Mr of 48,000. The other experimental conditions were the same as in the determination of the specific activities. The pH dependence of the k_{cat}/K_m was measured and analyzed according to a previous report.[15]

Results

Purification of Mutant Enzymes. To elucidate the role of the evolutionarily conserved residue in *Os*GSTF3, we substituted Tyr8 with Ala and Phe using oligonucleotide-directed mutagenesis. The mutant enzymes were expressed in *E. coli* under the control of the *tac* promoter. The expressed mutant enzymes were isolated and purified by affinity chromatography on immobilized GSH. The Y8F mutant was isolated in a yield of approximately 8 mg per liter of culture as in the case of the wild-type enzyme, showing that the

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutant	Sequence of primers[a]	Sense
Y8A	5'-GTGACGGTG<u>GC</u>CGGTCCGATG-3'	Forward
Y8F	5'-GTGACGGTGT<u>TT</u>GGTCCGATGATC-3'	Forward

[a]Changed bases are shown by underlines.

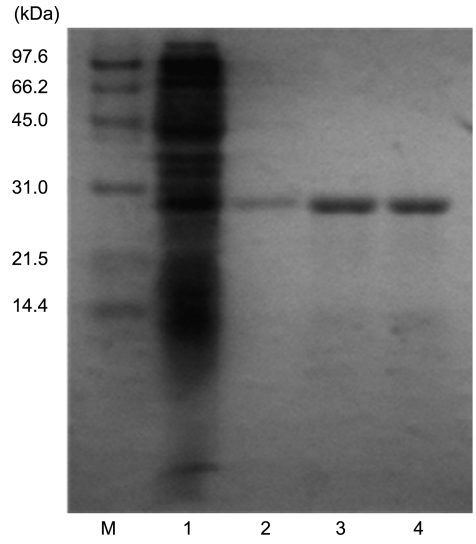

Figure 2. SDS-PAGE of mutant enzymes. Lane M, SDS low-range molecular weight marker; lane 1, purified Y8A; lane 2, purified Y8F; lane 3, purified *Os*GSTF3.

Table 2. Substrate specificity of *Os*GSTF3 and mutant enzymes

Substrate	Specific activity (μmol/min/mg)		
	*Os*GSTF3	Y8A	Y8F
CDNB	7.60 ± 0.08	0.88 ± 0.09	7.32 ± 0.09
CP	ND[a]	ND	ND
DCNB	ND	ND	ND
EPNP	0.48 ± 0.15	ND	0.42 ± 0.05
ETA	0.47 ± 0.02	ND	0.36 ± 0.06
NPB	ND	ND	ND

Values are Means ± S.D., generally based on n ≥ 5. [a]ND = Not detected

binding ability of the Y8F mutant to GSH-agarose was not much different from that of the wild-type enzyme. On the other hand, in the case of the Y8A mutant, the amount of the isolated enzyme was approximately 0.9 mg per liter of culture, showing the low affinity of the Y8A mutant for GSH-agarose. The purified mutant enzymes gave 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 Tyr8 Residue on Substrate Specificity. The substrate specificities of the mutant enzymes are shown in Table 2. The *Os*GSTF3 and the mutant enzymes showed no activity towards DCNB for Mu class GST, NPB for Theta class GST, and CP for glutathione peroxidase activity. The substitution of Tyr8 with Phe had a negligible effect on the GSH conjugation activity towards CDNB, EPNP, and ETA, demonstrating the non-essentiality of this residue. On the other hand, the substitution of Tyr8 with Ala showed a significant decrease (~90%) in the GSH-CDNB conjugation activity. Moreover, Y8A mutant also showed no activity towards EPNP and ETA.

Effects of Point Mutations of Tyr8 Residue 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 of Tyr8 with Phe scarcely affected the kinetic parameters. On the other hand, the substitution of Tyr8 with Ala significantly affected kinetic parameters. The K_m values of the Y8A mutant towards GSH and CDNB were approximately 1.6- and 2.6-fold larger than those of the wild type, respectively. Moreover, the Y8A mutant resulted in approximately 60-77% decrease of k_{cat} values.

Effect of Point Mutations of Tyr8 Residue on Dependence of k_{cat}/K_m^{CDNB} Values on pH. The enhancement of the nucleophilicity of the thiol group in GSH by lowering the pK_a value is considered to be a major role of GSTs in catalyzing the formation of GSH-conjugates. The k_{cat}/K_m^{CDNB} values of the wild-type enzyme and mutant enzymes for the GSH-CDNB conjugation were determined at various pH values with a large excess of GSH. The pH dependences of the k_{cat}/K_m^{CDNB} values of the wild type and the mutant enzymes are shown in Figure 3. From the plot of log (k_{cat}/K_m^{CDNB}) against pH, the pK_a values of the thiol group of GSH bound in the wild-type enzyme and Y8F mutant were approximately 6.8, whereas that in Y8A mutant was 8.5.

Discussion

The enhancement of the nucleophilicity of the thiol group of GSH by lowering its pK_a is considered to be a major role of GSTs in catalyzing the formation of GSH-conjugates.[2,3] The pK_a of the thiol group of GSH bound in hGST P1-1 is about 2.8 pK units lower than that of free GSH in aqueous solution.[15] Chemical modification and site-directed mutagenesis studies have suggested that tyrosine residues are located in the active site of GSTs.[16,17] The three-dimensional structure of GST also suggested the possibility that tyrosine residues could play an essential role in catalysis or substrate binding affinity.[9] These results have mainly been obtained from studies on the relationship between the structure and functions of mammalian GSTs.

In the present study, in order to elucidate the active site residues responsible for the catalytic properties of plant GSTs, we introduced site-directed mutations into Tyr8 of *Os*GSTF3 and examined the enzymatic properties of the mutated enzymes. It was found that the substitution of Tyr8 with Phe had a negligible effect on the GSH conjugation activity and kinetic parameters (Table 2 and 3). From the plot of log(k_{cat}/K_m^{CDNB}), the pK_a value of the thiol group of GSH bound in the Y8F mutant was also estimated to be the same as that of the wild-type, and the contribution of Tyr8 was supposed not to enhance the nucleophilicity of GSH (Fig. 3). From these results, we suggest that Tyr8 in *Os*GSTF3 is not an essential active-site residue. However, the substitution of this residue with Ala significantly decreased the catalytic activity of the enzyme (Table 2) and significantly affected K_m and k_{cat} values (Table 3). The pK_a value of the thiol group of GSH bound in the Y8A mutant enzyme-GSH complex was also estimated to be approximately 1.7 pK units higher than that in the wild-type enzyme-GSH complex

Table 3. Kinetic parameters of *Os*GSTF3 and mutant enzymes for GSH-[1-chloro-2,4-dinitrobenzene] conjugation

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 ($mM^{-1}s^{-1}$)
*Os*GSTF3	0.25 ± 0.08	4.01 ± 0.21	16.0	0.85 ± 0.07	5.48 ± 0.21	6.5
Y8A	0.39 ± 0.07	0.92 ± 0.10	2.4	2.20 ± 0.13	2.17 ± 0.18	1.0
Y8F	0.26 ± 0.04	3.82 ± 0.16	14.9	0.84 ± 0.06	5.26 ± 0.17	6.3

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

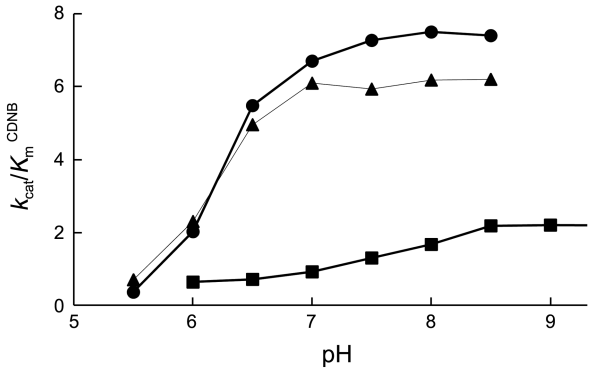

Figure 3. Dependence of k_{cat}/K_m^{CDNB} values on pH. The kinetic parameters of *Os*GSTF3, Y8F and Y8A for the conjugation of GSH with CDNB were determined under the conditions of saturating GSH (2.5 mM) and variable concentrations of CDNB (0.1-1.0 mM). -●-, *Os*GSTF3; -▲-, Y8F; -■-, Y8A.

(Fig. 3). These results suggested that the substitution of Tyr8 with Ala resulted in significant structural changes of the active site. Three dimensional structures of plant GSTs have suggested that the active-site Tyr residue of the mammalian Alpha, Mu, Pi, and Sigma GSTs is substituted with Ser or Cys residue.[18] Through preliminary data (studies are currently in progress) on the three-dimensional structure of *Os*GSTF3 in complex with GSH, the benzene ring of Tyr8 is oriented towards the GSH. On the other hand, the hydroxyl group of Ser13 is at the appropriate distance to form a hydrogen bond with the sulfur atom of GSH. Thus, the Tyr8 residue is expected not to participate directly in the catalytic mechanism, while the substitution of Tyr8 with Ala is also thought to affect the interaction between the thiol group of GSH and the hydroxyl group of the Ser13 through distorting the orientation of the GSH bound in the enzyme and/or the binding site of electrophilic substrates. Taken together, we suggest that Tyr8 residue in *Os*GSTF3 is not essential for the catalytic activity of lowering the pK_a of the thiol of GSH in the enzyme-GSH complex, but may contribute to the integrity of the conformation of the active site for the construction of the proper electrostatic field. This finding stresses the importance of structural residues that affect the enzymatic properties such as catalytic activity and kinetic parameters.

In conclusion, the presented results suggest that the evolutionarily conserved Tyr8 residue in *Os*GSTF3 seems to influence the structural stability of the active site in *Os*GSTF3 rather than directly the catalytic activity. Further clarification on the relationship between the structure and catalytic activity of these residues requires three-dimensional structure analysis of the mutant enzymes.

Acknowledgments. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2012R1A1B3000478).

References

1. Mannervik, B. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1985**, *57*, 357.
2. Mannervik, B.; Danielson, U. H. *CRC Crit. Rev. Biochem.* **1988**, *23*, 283.
3. Fahey, R. C.; Sundquist, A. R. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1991**, *64*, 1.
4. Dixon, D. P.; Lapthorne, A.; Edwards, R. *Genome Biol*. **2002**, *3*, 3004.1.
5. Dixon, D. P.; Cole, D. J.; Edwards, R. *Plant Mol. Biol*. **1998**, *36*, 75.
6. Dixon, D. P.; Davies, B. G.; Edwards, R. *J. Biol. Chem*. **2002**, *277*, 30859.
7. Edwards, R.; Dixon, D. P.; Walbot, V. *Trends Plant Sci*. **2000**, *5*,193.
8. Droog, F. *J. Palt Growth Regul*. **1997**, *16*, 95.
9. Board, P. G.; Menon, D. *Biochim. Biophys. Acta* **2013**, *1830*, 3267.
10. Cho, H.-Y.; Kong, K.-H. *Pestic. Biochem. Phys*. **2005**, *83*, 29.
11. Yoon, S.-Y.; Kong, J.-N.; Jo, D.-H.; Kong, K.-H. *Food Chem*. **2011**, *129*, 1327.
12. Jo, H.-J.; Lee, J.-W.; Noh, J.-S.; Kong, K.-H. *Bull. Korean Chem. Soc*. **2012**, *33*, 4169.
13. Habig, W. H.; Jakoby, W. B. *Methods Enzymol*. **1981**, *77*, 398.
14. Flohè, L.; Günzler, W. A. *Methods Enzymol*. **1984**, *105*, 114.
15. Kong, K.-H.; Takasu, K.; Inoue, H.; Takahashi, K. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 194.
16. Kong, K.-H.; Nishida, M.; Inoue, H.; Takahashi, K. *Biochem. Biophys. Res. Commun*. **1992**, *182*, 1122.
17. Barycki, J. J.; Colman, R. F. *Biochemistry* **1993**, *32*, 13002.
18. Cummins, I.; Dixon, D. P.; Freitag-Pohl, S.; Skipsey, M.; Edwards, R. *Drug Metab. Rev.* **2011**, *43*, 266.