ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 10, No. 5, 1997
Printed in the Republic of Korea

Functional analysis of Tyr7 residue in human glutathione *S*-transferase P1-1

Kwang-Hoon Kong, Hee-Joong Park, Suck-Young Yoon and Sung-Hee Cho
Department of Chemistry, College of Sciences, Chung-Ang University, Dongjak-ku, Seoul 156-756, Korea
(Received June 16, 1997)

Human glutathione *S*-transferase 중 tyrosine 7 잔기의 기능 분석

공광훈 · 박희중 · 윤석영 · 조성희
중앙대학교 화학과
(1997. 6. 16. 접수)

Abstract: In order to clarify the functional role of Tyr7 in human glutathione *S*-transferase P1-1, we extensively investigated the effect of mutation of Tyr7 on the substrate specificity and inhibition characteristics. The mutational replacement of Tyr7 with phenylalanine lowered the specific activities with 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy) propane for GSH-conjugation reaction to 3~5% of the values for the wild-type enzyme. The pK_a of the thiol group of GSH bound in Y7F was about 2.4 p*K* units higher than that in the wild-type enzyme. The I_{50} of hematin for Y7F was similar to that for the wild-type enzyme and those of benastatin A and *S*-(2,4-dinitrophenyl)glutathione were only moderately decreased. These results suggest that Tyr7 is considered to be important the catalytic activities not only for GSH-chloronitrobenzene derivatives but also for GSH-epoxide conjugation reaction, rather than to binding of the substrates.

요약: 본 실험은 human glutathione *S*-transferase P1-1의 tyrosine 7 잔기에 대한 변이체를 작성하고, 기질특이성과 저해제의 효과를 조사하여, 이 잔기의 기능을 분석한 것이다. 1,2-dichloro-4-nitrobenzene과 1,2-epoxy-3-(*p*-nitrophenoxy)propane에 대한 GSH 포합반응에 대한 활성은 야생형에 비해 변이체 Y7F에서는 3~5%로 크게 저하하였으며, 효소에 결합한 GSH의 thiol기의 p*K*a 는 2.4 p*K* 높았다. 저해제 hematin에 대한 I^{50}값은 야생형과 변이체 Y7F에서 비슷하게 나타났으며, 저해제 benastatin A와 *S*-(2,4-dinitrophenyl) glutathione에 대한 I^{50}값들은 다소 감소하였다. 이러한 결과들로부터 tyrosine 7 잔기는 기질의 결합에 관여하기보다 GSH-chloronitrobenzene 유도체와 GSH-epoxide 포합반응에 대한 촉매활성에 중요하다고 생각된다.

Key words: glutathione *S*-tranferase, tyrosine 7 residue, active site, substrate specificity, inhibition characteristics.

1. Introduction

Glutathione *S*-transferase(GST, EC 2. 5. 1. 18) is a family of multifunctional enzymes, which consist of homo- or heterodimers of subunits whose molecular weights are about 25 kDa.[1,2] They catalyze the formation of conjugates between reduced glutathione(GSH) and a wide variety of

reactive compounds that could react with nucleophilic chemical groups in proteins and nucleic acids and thus cause toxic effect, mutations and cancer. They can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity.[3] They are distributed in a wide range of organisms from mammal to *E. coli*.[4] They can be grouped into at least four distinct classes, alpha, mu, pi, and theta according to their structures and catalytic properties.[5]

The active site of GST is suggested to consist of a GSH-binding site (G-site) and a nonspecific hydrophobic site (H-site) to accommodate the electrophilic substrates. Although the G-site and the catalytic mechanism of this enzyme have been the targets of many investigations involving chemical modification,[6~9] site-directed mutagenesis[10~13], they have remained unclear for a long time. Chen *et al.* mentioned that one major contribution of GST to catalysis is to lower the p*K*a of the bound nucleophile, GSH.[14] The participation of a general base in the catalytic mechanism is expected and its p*K*a is estimated to be>7.5 in rat GST 4-4 and 7.6 in rat GST 3-3.[14,15] However, the essentiality of a histidine residue(s) as a general base in the catalytic mechanism of GSH-conjugating reaction by human class Pi GST was rejected by the study using site-directed mutagenesis.[10]

By chemical modification study, tyrosine residues have been suggested to be present at or near the active site of GST.[16] Human GST P1-1 was inactivated by incubation with *N*-acetylimidazole, an *O*-acetylating reagent for tyrosine residues. The inactivation of the enzyme by *N*-acetylimidazole was effectively blocked in the presence of GSH at 0.1~1mM, which was much lower than concentration of the acetylating reagent(6mM), whereas it was not blocked at all in the presence of 1-chloro-2,4-dinitrobenzene. This result suggested that the acetylated residue that was responsible for the inactivation was located in the GSH-binding site of the enzyme. Twelve tyrosine residues are present in hGST P1-1. Among them, only Tyr7 is conserved in all cytosolic GSTs(*Fig.* 1). Moreover, the three-dimensional structure of the *S*-hexyl GSH-bound class Pi GST, reported by Reinemer *et al.*, shows that the *S*-hexyl group is located adjacent to the hydroxyl group of Tyr7.[17] Accordingly, the thiol group of GSH bound to the GST is also expected to lie in the immediate neighborhood of the hydroxyl group of Tyr7.

GSTs	Sequences
Class Pi	7[a]
1. Human	PPYTVVYFPVRGRC
2. Pig	PPYTITYFPVRGRC
3. Rat	PPYTIVYFPVRGRC
4. Mouse	PPYTIVYFPVRGRC
Class Alpha	
5. Rat Ya(a)	SGKPVLHYFNARGRM
6. Rat Ya(b)	SGKPVLHYFNARGRM
7. Rat Yc	PGKPVLHYFDGRGRM
8. Rat 8	EVKPKLYYFQGRGRM
9. Mouse Ya	AGKPVLHYFNARGRM
10. Mouse Yc	AGKPVLHYFDGRGRM
11. Rabbit alpha I	ARKPLLHYFNGRGRM
12. Rabbit alpha II	AGKPKLHYFNARGRM
13. Human Ha-1	AEKPKLHYFNARGRM
14. Human Ha-2	AEKPKLHYSNIRGRM
15. Chick CL3	AAKPVLYYFNGRGKM
Class Mu	
16. Rat Yb1a	PMILGYWNVRGLT
17. Rat Yb1b	PMILGYWNVRGLT
18. Rat Yb2	PMTLGYWDIRGLA
19. Rat Yb3	PMTLGYWDIRGLA
20. Rat Yn4	AMILGYWNVRGLT
21. Hamster	PVTLGYWDIRGLA
22. Murine mGTmu1	PMILGYWNVRGLT
23. Murine mGTmu2	PMTLGYWDIRGLA
24. Murine GT55a	PMTLGYWNTRGLT
25. Human GTHMUS	PMTLGYWNIRGLA
26. Human GST1-1	PMILGYWDIRGLA
27. Human GST1-2	PMILGYWDIRGLA
28. Human HTGT6	CESSMVLGYWDIRGLA
29. Schistosoma japonicum	TKLPILGYWKIKGLV
Class Thata	
30. Rat Yrs	GLELYLDLLSQP
Insect	
31. Musca domestica	DFYYLPGSAP
32. Drosophila 1-1	VDFYYLPGSSP
Plant	
33. Maize I	APMKLYGAVMSWN
34. Maize III	APLKLYGMPLSPN
Bacteria	
35. *E.coli*	MKLFYKGGACSL
36. *Proteus*	YYTGSSPHV

Fig. 1. Evolutionally conserved tyrosine residue.[26,32]

In this study, we investigated the substrate specificity and the inhibitory effect of the wild-type enzyme and Y7F mutant in order to clarify the functional role of Tyr7.

2. Materials and Methods

2.1. Materials

Wild-type hGST P1-1 was obtained by expression of a cloned cDNA in *E. coli* as described in the previous paper.[10,18] GSH, 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were purchased from Kohjin Co. and Wako Pure Chem. Ind., respectively. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane was obtained from Sigma. Glutathione-agarose was prepared as described by Simons and Vander Jagt.[19] *S*-(2,4-dinitrophenyl) glutathione was synthesized by the method of Schramm *et al.*[20] Benastatin A was gifted by Prof. Takaaki Aoyagi.[21]

2.2. Preparation of mutant enzyme

Synthesis of the oligonucleotide, site-directed mutagenesis, confirmation of mutation, construction of the expression plasmid, expression and purification of the mutant enzyme were performed as described in the previous paper.[16] Protein concentration of the wild-type enzyme was determined by measuring the absorbance at 280nm as described by Parker[22] and protein concentration of the mutant was determined by using protein assay reagent(Bio-Rad Lab.) and the wild-type enzyme as a standard protein.

2.3. Enzyme activity

The specific activities were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with 1,2-dichloro-4-nitrobenzene or 1,2-epoxy-3-(*p*-nitrophenoxy)propane as described by Habig and Jakoby.[23] A unit of activity 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 units of enzyme activity per mg of protein.

2.4. Inhibition studies

The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1mM GSH and the inhibitor for 2 min and initiating the reaction by addition of 20μL of 50mM 1-chloro-2,4-dinitrobenzene(final concentration, 1mM). The concentration of inhibitor giving 50% inhibition(I_{50}) was determined from plot of residual activity against inhibitor concentration.

2.5. Electrophoresis and blotting

Denaturing SDS-PAGE was carried out by the method of Laemmli[24] in 12.5% gels. The molecular-mass makers were SDS molecular weight standard markers(Bio-Rad) that contains phosphorylase B(92.5kDa), bovine serum albumin(66.2kDa), ovalbumin(45.0kDa), carbonic anhydrase (31.0kDa), soybean trypsin inhibitor(21.5kDa) and lysozyme 14.4kDa). Coomassie Blue R-250 was used for staining. Electrophoretic transfer of proteins to transfer PVDF-membrane(Millipore) in 2.5mM Tris base, 192mM glycine, 20% methanol from 12.5% SDS polyacrylamide gel was carried out by the method of Towbin *et al.*[25] Electroblotting was carried out for 90min with 150 mA. Blotted proteins were detected with primary antiserum for hGST P1-1 and a secondary peroxidase rabbit IgG of Vectastain ABC kit(Vector Lab. Inc.).

2.6. The dependence of kinetic parameters on pH.

The dependence of k_{cat}/K_m^{CDNB} on pH was determined by using the following buffers(0.1M) at the incubated pH: Bis-Tris-HCl, from 5.5 to 7.0; Tris-HCl, from 7.5 to 9.0; sodium 3-cyclohexylaminopropanesulfonate, 9.5. Reaction was carried out at saturating concentration of GSH(2.5mM) for the enzyme activity and variable CDNB.

2.7. Circular dichroism analysis.

Circular dichroism spectra were taken on a Jasco J-600 spectropolarimeter. Circular dichroic spectra of enzymes were recorded in 20mM potassium phosphate buffer(pH 7.0) using a 1mm cuvet path-length. The measurements are expressed as mean residue ellipticity, $[\theta]_{Mr}$, with a mean residue weight(Mr) of 110 for hGST P1-1. All measurements were made at 30℃.

2.8. Amino acid sequence analysis.

Amino acid sequencing was performed by using an automated protein sequencer, model 477A, equipped with on-line HPLC(Applied biosystems, Inc.).

3. Results and discussion

All the known mammalian cytosolic GSTs are homo- or hetero-dimers of subunits that consist of 200-240 amino acid residues. Only about 5% of the amino acid residues are conserved in all cytosolic GSTs.[3] The six residues are conserved in all the known sequences of GSTs: those are Tyr7, Pro53, Asp57, Ile68, Gly145 and Asp152 in hGST P1-1. Highly conserved residues are expected to be important for catalytic activity. However, Asp57 and Asp152 are not important for catalytic activity of GST.[26] Among the other four conserved residues, only Tyr7 can be a major candidate of catalytic residue(*Fig.* 1).

3.1. Purification and PAGE of mutant enzymes

To investigate the roles of the evolutionally conserved Tyr7 in hGST P1-1 was replaced with phenylalanine that is isosteric and uncharged by oligonucleotide-directed mutagenesis. The mutant hGST P1-1 expressed in *E. coli* under the control of *tac* promoter was isolated and purified by affinity chromatography on immobilized GSH. The affinity of the enzyme was not lowered by the replacement of Tyr7 with phenylalanine. One liter of culture yielded approximately 3mg each of the purified wild type and Y7F mutant. The purified wild type and Y7F mutant appeared as a single band on SDS-PAGE with an apparent Mr of 25,000(*Fig.* 2) and the antibody against the purified wild-type hGST P1-1 prepared from *E. coli* recognized Y7F mutant specifically(*Fig.* 3). The N-terminal amino acid sequence of the purified Y7F was confirmed by amino acid sequencing to be PPYTVVFFPVR.

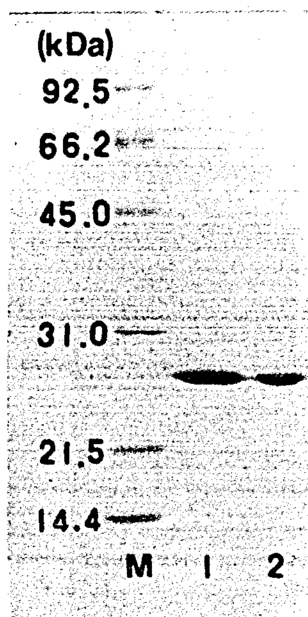

Fig. 2. Electrophoresis of recombinant hGST P1-1 and Y7F under denaturing conditions: Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gel. Coomassie blue R-250 was used for staining. Lane M, molecular mass maker(Bio-Rad); lane 1, wild-type enzyme; lane 2, Y7F.

3.2. Substrate specificity

It has been shown that the initial step in mercapturic acid formation is conjugation of the foreign compound with GSH, a reaction catalyzed by GSTs for many substrates.[1] The GSH-1,2-dichloro-4- nitrobenzene conjugating activities of the wild type and Y7F were assayed(*Table* 1). The specific activity of the wild-type enzyme toward 1, 2-dichloro-4-nitrobenzene was 0.118 μmol/min/mg. On the other hand, the specific activity of Y7F

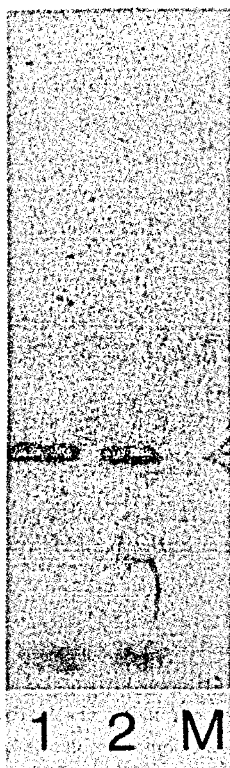

Fig. 3. Western blot analysis of the purified hGST P1-1 and Y7F: Lane M, molecular mass maker(Bio-Rad): lane 1, wild-type enzyme: lane 2, Y7F.

toward 1,2-dichloro-4-nitrobenzene was a 3.6% of that of the wild-type enzyme.

Epoxides are substrates that may be derivatives of naturally occurring compounds as well as of xenobiotics and are known as mutagenic and carcinogenic substances. The GSH-[1,2-epoxy-3-(*p*-nitrophenoxy)propane] conjugating activities of the wild-type enzyme and Y7F were assayed(*Table* 1). The specific activity of the wild-type enzyme toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane was 0.360μmol/min/mg. On the other hand, the specific activity of Y7F toward 1,2-epoxy-3-(p-nitrophenoxy)propane was about 5.1% of that of the wild-type enzyme. This result suggests that the hydroxyl group of Tyr7 is responsible for GSH-conjugation reaction with epoxide. Ji *et al.* reported that the hydroxyl group of Tyr115 in rat liver GST M3-3 also was an electrophilic participant in the addition of GSH to epoxides, and the two tyrosine residues(Tyr6 and Tyr115, the counterparts of Tyr7 and Tyr108 in hGST P1-1, respectively) represent a classical push-pull catalytic ensemble in this reaction.[27]

3.3. Inhibition studies

The inhibitory effects on the GSH-[1-chloro-2,4-dinitrobenzene] conjugating activity of various kinds of inhibitors, benastatin A, hematin and *S*-(2,4-dinitrophenyl)glutathione were determined under the standard assay conditions(*Table* 2). The I_{50} of benastatin A, an electrophilic substrate-like compound,[21] for Y7F was lower than that for the wild-type enzyme about half-fold. The I_{50} of *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with 1-chloro-2,4-dinitrobenzene, for Y7F was lower than that for the wild-type enzyme about half-fold. The I_{50} of hematin, a non-substrate ligand, for Y7F was similar to that for the wild-

Table 1. Specific activities of the wild-type enzyme and Y7F mutant for GSH-conjugation with 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane

Enzymes	1,2-dichloro-4-nitrobenzene		1,2-epoxy-3-(*p*-nitrophenoxy)propane	
	Specific activity (μmol/min/mg)	Relative activity (%)	Specific activity (μmol/min/mg)	Relative activity (%)
Wild type	0.118±0.011	100	0.360±0.038	100
Y7F	0.004±0.001	3.4	0.019±0.001	5.3

Values are means±S.D., generally based on n≥5.

Table 2. Inhibitory effect of benastatin A, S-(2,4-dinitrophenyl)glutathione and hematin on GSH-[1-chloro-2, 4-dinitrobenzene] conjugation

Inhibitors	wild type		Y7F mutant	
	$I_{50}(\mu M)$	%	$I_{50}(\mu M)$	%
Benastatin A	2.7±0.11	100	1.3±0.1	48
S-(2,4-dinitrophenyl)GSH	13.5±1.2	100	7.5±0.6	56
Hematin	0.62±0.02	100	0.59±0.02	95

Values are means±S. D., generally based on n≥5.

type enzyme, indicating that tyrosine 7 is not involved in heme binding.

3.4. Dependence of kinetic parameters on pH

The k_{cat}/K_m^{CDNB} values of the wild-type enzyme and Y7F for GSH-CDNB conjugating reaction were determined at various pH with a large excess of GSH. The pH dependences of k_{cat}/K_m^{CDNB} of the wild-type enzyme and Y7F are shown in *Fig.* 4. From the plots of $\log(k_{cat}/K_m^{CDNB})$ against pH, the pKa values of the thiol group of GSH bound in wild-type enzyme and Y7F were estimated to be approximately 6.3 and 8.7, respectively. No gross change in secondary structure was suggested from comparison of CD spectra of the wild-type enzyme and Y7F(*Fig.* 5). Enhancement of the nucleophilicity of the thiol group in GSH by lowering its pKa is considered to be a major role of GSTs in catalyzing the formation of GSH-conjugate.[14,15] The pKa of the thiol group of GSH bound in hGST P1-1 was about 2.8-pK units lower than that of free GSH in aqueous solution. However, the replacement of Tyr7 with phenylalanine increased the pKa

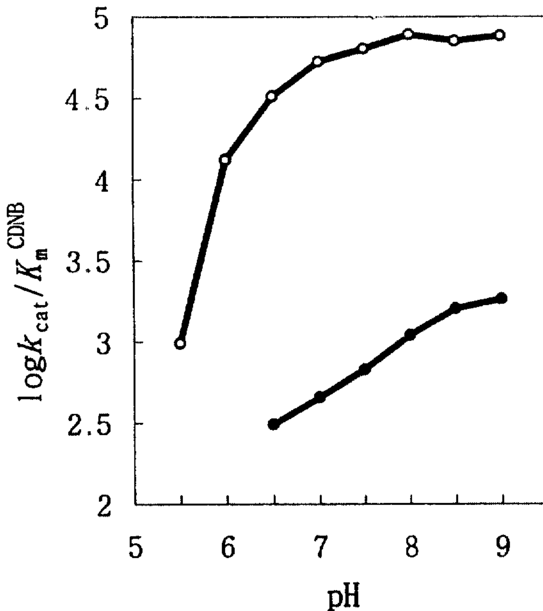

Fig. 4. Dependence of k_{cat}/K_m^{CDNB} values on pH: The kinetic parameters of the mutants for the conjugation of GSH with CDNB were determined under the conditions of GSH(2.5mM) and variable concentrations of CDNB(0.2~1.0mM) in the following buffers (100mM) at the indicated pH: Bis-Tris-HCl, from 5.5 to 7.0; Tris-HCl, from 7.5 to 9.0. ○, wild type ; ●, Q64A.

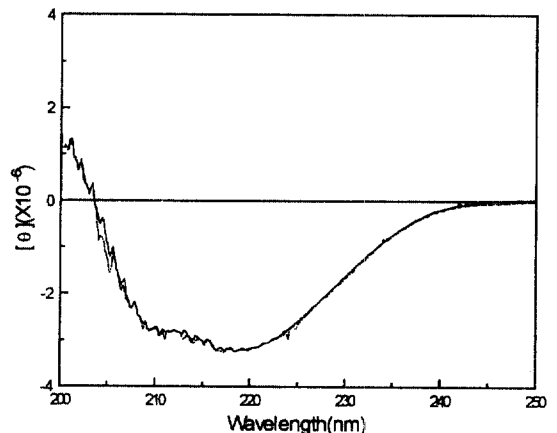

Fig. 5. Circular dichroism spectra of hGST P1-1 wild-type and Y7F mutant enzyme at far-ultraviolet region: The spectra were recordered in 20mM potassium phosphate buffer(pH 7.0) using a 1mm cuvet pathlength. —, wild-type enzyme; ···, Y7F.

of the thiol group of enzyme-bound GSH about 2.4 p*K* units. The interaction between the hydroxyl group of Tyr7 and the thiol group was essential to GSH-conjugation reaction through lowering p*K*a of the thiol. This fact was supported by site-directed mutagenesis of the class Alpha and Mu GSTs,[11] and by the three-dimensional structure of the hGST P1-1 in complex with *S*-hexyl-GSH,[17] and the pGST P1-1 in complex with glutathione sulfonate suggesting that the *S*-hexyl group and sulfonate group are located adjacent to the hydroxyl group of Tyr7.[28] GST also appears to provide for the partical desolvation of the thiolate anion in the active site. In fact, pH-rate profiles show that even at high pH the Y7F mutant is about 25-fold less efficient than the wild-type hGST P1-1, suggesting that Tyr7 serves an additional function. A preliminary examination of the crystal structure of the Y6F mutant of rGST M3-3(the counterpart of Y7F mutant of hGST P1-1) in complex with GSH indicates that the geometry of the active site is essentially unchanged with respect to the wild-type enzyme but that an additional water molecule occupies the solvation shell of the sulfur.[26] Therefore, it may be that hydroxyl group of Tyr7 serves as part of a surrogate solvation shell in the hydrophobic active site or that it helps to orient the thiolate anion correctly in the active site.

Surprisingly, it is suggested that this apparently conserved tyrosine residue in *Escherichia coli* GST is not essential for catalytic activity by site-directed mutagenesis study.[29] Crystal structure of a theta-class GST from *Lucilia cuprina* also shows that the equivalent residue in the theta- class structure is not in the active site, but its role appears to have been replaced by either a nearby serine or by another tyrosine residue located in the C-terminal domain of the enzyme.[30] To explain these results the following possibility can be considered that *E. coli* and theta-class GSTs have acquired somewhat different catalytic mechanism or three- dimensional structure in the process of evolution, and some other residue than the tyrosine residue near the N terminus or another tyrosine residue in another part of the molecule may participate in catalysis in *E. coli* and theta-class GSTs. To clarify this problem, it seems to be essential to extend further extensive studies on the structure and function relationships.

4. Conclusions

It is quite clear from this study that Tyr7 is essential to catalytic activity. Removal of the hydroxyl group of Tyr7 has no significant effects on I_{50} values of inhibitors. Nevertheless the Y7F mutant exhibits specific activities of only about 3~5% in comparison with those of the wild-type enzyme. Consequently, the hydroxyl group of Tyr7 is considered to participate in both GSH-chloronitrobenzene derivatives and GSH-epoxide conjugation reaction.

Acknowledgement

This research is supported by the Korean Ministry of Education through Research Fund.

References

1. Mannervik, B., *Adv. Enzymol. Rel. Areas Mol. Biol.* **57**, 357-417(1985).
2. Pickett, C. B. and Lu, A. Y. H., *Annu. Rev. Biochem,* **58**, 734-764(1989).
3. Mannervik, B. and Danielson, U. H., *CRC Crit. Rev. Biochem.* **23**, 283-337(1988).
4. Fahey, R. C. and Sundquist, A. R., *Adv. Enzymol. Rel. Areas Mol. Biol.,* **64**, 1-53(1991).
5. Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M. and Wolf, C. R., *Biochem. J.,* **282**, 305-306(1992).
6. Desideri, A., Caccuri, A. M., Poligio, F., Bastoni, R.

and Federici, G., *J. Biol. Chem.*, **266**, 2063-2066 (1991).

7. Lo Bello, M., Petruzzelli, R., De Stefano, E., Tenedini, C., Barra, D. and Federici, G., *FEBS Lett.* **263**, 389-391(1990).
8. Nishihara, T., Maeda, H., Okamoto. K., Oshida, T., Mizoguchi, T. and Terada, T., *Biochem. Biophys. Res. Commun.*, **174**, 580-585(1991).
9. Tamai, K., Satoh, K., Tsuchida, S., Hatayama, I., Maki, T. and Sato, K., *Biochem. Biophys. Res. Commun.* **167**, 331-338(1990).
10. Kong, K. -H., Inoue, H. and Takahashi, K., *Biochem. Biophys. Res. Commun.*, **181**, 748-755(1991).
11. Stenberg, G., Board, P. G. and Mannervik, B., *FEBS Lett.*, **293**, 153-155(1991).
12. Wang, J. -Y., McCommas, S. and Syvanen, M., *Mol. Gen. Genet.*, **227**, 260-266(1991).
13. Zhang, P., Graminski, G. F. and Armstrong, R. N., *J. Biol. Chem.*, **266**, 19475-19479(1991).
14. Chen, W. -J., Graminski, G. F. and Armstrong, R. N., *Biochemistry.*, **27**, 647-654(1988).
15. Graminski, G. F., Kubo, Y. and Armstrong, R. N., *Biochemistry*, **28**, 3562-3568(1989).
16. Kong, K. -H., Nishida, M., Inoue, H. and Takahashi, K., *Biochem. Biophys. Res. Commun.*, **182**, 1122-1129(1992).
17. Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G. and Parker, M. W., *J. Mol. Biol.* **227**, 214-226(1992).
18. Kong, K. -H., Takasu, K., Inoue, H. and Takahashi, K., *Biochem. Biophys. Res. Commun.*, **184**, 194-197 (1992).
19. Simons, P. C. and Van der Jagt, D. L., *Anal. Biochem.*, **82**, 334-341(1977).
20. Schramm, V. L., McCluskey, R., Emig, F. A. and Litwack, G., *J. Biol. Chem.*, **259**, 714-722(1984).
21. Aoyagi, T., Aoyama, T., Kojima, F., Matsuda, N., Maruyama, M., Hamada, M. and Takeuchi, T., *J. Antibiot.*, **45**, 1385-1390(1992).
22. Parker, M. W., Bello, M. L. and Federici, G., *J. Mol. Biol.*, **213**, 221-222(1990).
23. Habig, W. H. and Jakoby, W. B., *Methods Enzymol.*, **77**, 398-405 (1981).
24. Laemmli, U. K., *Nature*(London), **227**, 680-685 (1970).
25. Towbin, H., Staehelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354(1979).
26. Kong, K. −H., Inoue, H. and Takahashi, K., *Protein Eng.*, **6**, 93-99(1993).
27. Ji, X., Johnson, W. W., Sesay, M. A., Dickert, L., Prasad, S. M., Ammon, H. L., Armstrong, R. N. and Gilliland, G. L., *Biochemistry*, **33**, 1043-1052(1994).
28. Reinemer, P., Dirr, H. W., Ladenstein, R., Sch ffer, J., Gally, O. and Huber, R., *EMBO J.*, **10**, 1997-2005 (1991).
29. Nishida, M., Kong, K. -H., Inoue, H. and Takahashi, K., *J. Biol. Chem.*, **269**, 32536-32541 (1994).
30. Wilce, M. C. J., Board, P. G., Feil, S. C. and Parker, M. W., *EMBO J.*, **14**, 2133-2143(1995).