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Substitutions for Cys-472 and His-509 at the Active Site of β-Galactosidase from *Lactococcus lactis* ssp. *lactis* 7962 Cause Large Decreases in Enzyme Activity

CHUNG, HYE YOUNG, EUN JU YANG, AND HAE CHOON CHANG*

Department of Food and Nutrition, Chosun University, Gwangju 501-759, Korea

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Abstract Structural modeling of β-galactosidase from *L. lactis* ssp. *lactis* 7962 has shown that the residues Cys-472 and His-509 are located in the wall of the active-site cavity. To examine the functions of Cys-472 and His-509, we generated five site-specific mutants: Cys-472-Ser, Cys-472-Thr, Cys-472-Met, His-509-Asn, and His-509-Phe. β-Galactosidase substituted at Cys-472 with Met or His-509 with Phe had <3% of the activity of the native enzyme when assayed using ONPG as substrate. The other mutants Cys-472-Ser, Cys-472-Thr, and His-509-Asn had *ca.* 10–15% of the native enzyme activity. The V_{max} values of the five mutated enzymes were lower (60–7,000-fold) than that of native enzyme. These results show that the catalytic ability of β-galactosidase is significantly affected by mutations at Cys-472 or His-509.

Key words: β-Galactosidase, Cys-472, His-509, *L. lactis* 7962

Historically, the traditional roles for many lactic acid bacteria (LAB) has been as starter cultures to drive fermented food and feed products. In recent years, there has been major advances in the genetic information of LAB [2, 5, 6, 13, 16]. LAB metabolize lactose by using two different enzymes, phospho-β-galactosidase (P-β-gal) and β-galactosidase (β-gal) [2, 16]. P-β-gal is found in the majority of *Lactococcus lactis* strains. Unlike other lactococci, *L. lactis* ssp. *lactis* 7962 contains β-gal as its major lactose-utilizing enzyme. The complete *gal/lac* operon genes of *L. lactis* 7962 were cloned, and genetic studies of the β-gal gene were performed by our group [1, 6, 7].

The deduced amino acids sequence of the *L. lactis* 7962 β-gal gene (U60828) was compared with those of other microorganisms, and *E. coli* β-gal was found to have the highest (49.9%) identity [18]. Based on the X-ray crystallographic structure of *E. coli* enzyme [4], we previously carried out a structural design of the β-gal from *L. lactis* 7962 [13, 18]. We tried to explain the roles of the residues in β-gal from *L. lactis* 7962 by site-directed mutagenesis, based on the results of this structural modeling. It was suggested that Glu-384 and Glu-429 were probably ligands of Mg^{++}, and that the three-dimensional disposition of Mg^{++} and of its neighborhood interactions (Glu-384, Glu-429, Asp-428, or His-386) were important for the functional maintenance of β-gal [13]. A large reduction in catalytic efficiency was observed when structurally conservative replacements were made at Tyr-475 and Glu-506 of β-gal. It was concluded that Tyr-475 and Glu-506 were probably essential parts of the catalytic machinery at its active site [18].

The three-dimensional structure of *E. coli* β-gal shows that its substrate binding site lies in a deep pocket within a distorted "TIM" barrel. The residues Tyr-503, Glu-537, Cys-500, and His-540 are close together and are located around a deep pit at the end of the TIM barrel [4]. The residues Tyr-475, Glu-506, Cys-472, and His-509 in *L. lactis* 7962 β-gal are homologous with Tyr-503, Glu-537, Cys-500, and His-540 of *E. coli* β-gal, respectively.

The present study was undertaken to determine the importance of the interaction between His-509 and Cys-472, which should be located near Tyr-475 and Glu-506 in the active-site of β-gal from *L. lactis* 7962.

Structural Design and Mutagenesis

Based on the X-ray structure of *E. coli* β-gal [4], the secondary and tertiary structures of β-gal (*L. lactis* 7962) were predicted using the Nnpredict and Sybyl Version 6.3 program (Tripos Inc., St. Louis, MO, U.S.A.). Computer design of the structure of *L. lactis* 7962 β-gal indicated that Tyr-475 and Glu-506 are positioned at the substrate-

*Corresponding author
Phone: 82-62-230-7345; Fax: 82-62-222-8086;
E-mail: hcchang@mail.chosun.ac.kr

Table 1. Amino acid sequences of several microorganisms showing homology in the region equivalent to the active site of *Lactococcal* 7962 β-galactosidase.

M/O \ 7962 aa residue	384	386	428	429	472	475	506	509
L. lactis 7962	E	H	N	E	C	Y	E	H
E. coli lacZ	E	H	N	E	C	Y	E	H
Kl. pneumoniae	E	H	N	E	C	Y	E	H
B. megaterium	E	H	N	E	S	Y	E	H
Lb. bulgaricus	E	H	N	E	S	Y	E	H
Bif. longum	E	H	N	E	T	Y	E	H
Kl. lactis	E	H	N	E	S	Y	E	H
S. thermophilus	E	H	N	E	S	Y	E	H
E. coli ebgA	E	H	N	E	S	Y	E	H
C. acetobutylicum	E	H	N	E	S	Y	E	H
Arthrobacter sp.	E	H	N	E	S	Y	E	H
Leu. lactis	E	H	N	E	S	Y	E	H
L. acidophilus	E	H	N	E	S	Y	E	H
Reference	[10]	[10]	[10]	[10]	This study	[7]	[7]	This study

binding site. We previously reported that these act as functional residues at the substrate-binding site [18]. The sequences of β-gals from different sources demonstrate that the residues that form the active-site pocket are highly conserved [4]. According to the X-ray structure of *E. coli* β-gal and comprehensive review of the active site and mode of action of β-gal from *E. coli* [3, 4, 12], it was predicted that the active site of *L. lactis* 7962 β-gal incorporates His-509 and Cys-472 and that these are located in the wall of the active-site cavity.

We mutated six sites (Glu-384, His-386, Asp-428, Glu-429, Tyr-475, and Glu-506) in *L. lactis* 7962 β-gal in our previous study. These sites have been shown to be important conserved sites for enzyme function, such as for Mg^{++} ligand [10] or for substrate binding [18] (Table 1). However, in the present study, Cys-472, which should be positioned at an active site, was not found to be conserved. It was reported by Moon *et al.* [10] that somewhat surprisingly, a high level of conservation in β-gal does not always imply functional importance. Nevertheless, it is possible that such amino acids are important for structural stability.

To determine the importance of His-509 and Cys-472 in the substrate-binding pocket, we adopted a site-directed mutagenesis technique [8, 14]. Mutated β-gal was generated by using PCR-based site-directed mutagenesis (Exsite PCR-Based Site-Directed Mutagenesis Kit; Stratagene), according to the manufacturer's instructions. Mutagenic reaction conditions were modified slightly according to the primer sequences. The mutagenic primer sequences used in this experiment are listed in Table 2. The *Sal*I and *Pst*I sites were generated at each end of the β-gal gene from *L. lactis* 7962 by PCR. A PCR-generated β-gal gene fragment was ligated into pBluescript KS^- (pBluescript+β-gal) and transformed into *E. coli*. The β-gal so expressed was considered to be the native enzyme and all the mutated β-gals were generated from its gene. His-509 was replaced with Asn and Phe. Asn was introduced because the amido group can form H bonds similar to those of His, and because the distance between the α-carbon of His and the ^{1}N of His is similar to the distance between the α-carbon of Asn and the amido nitrogen of Asn. Phe was introduced to eliminate the possibility of H bond formation, because Phe

Table 2. The mutagenic oligonucleotide sequences used in this experiment.

Primers	Sequences	Length (bp)
Cys-472-Ser	5'-TGATATCATT**TCT**CCGATGTATG-3' Ser	27
Cys-472-Thr	5'-TGATATCATT**ACT**CCGATGTATG-3' Thr	27
Cys-472-Met	5'-TGATATCATT**ATG**CCGATGTATGCTAGAG-3' Met	29
His-509-Asn	5'-GAATATGCT**AAT**GATATGGGTATTTC-3' Asn	26
His-509-Phe	5'-GAATATGCT**CAT**GATATGGGTATTTC-3' Phe	26

and His are approximately of the same size, and because His has an aromatic character. Cys-472 was replaced with Ser, Thr, or Met. Ser is similar to Cys but has a hydroxyl residue (-OH) instead of a sulfhydryl residue (-SH), whereas Thr is almost identical structurally to Ser, but contains an additional $-CH_3$ residue. Met was introduced to eliminate the possibility of H bond formation, to mask the -SH residue of Cys by $-S-CH_3$, and because Met occupies a larger space in the wall of the active-site cavity than Cys.

Expression of the Mutated β-Gal

For mutant screening, the β-gal activities of *E. coli* transformants harboring mutated β-gal genes were measured by using a modification of the Miller method [13, 18]. Briefly, 1 ml of the cells was harvested, washed twice with Z-buffer, and resuspended in Z-buffer. The resuspended cell volume was adjusted to 1 ml, and mixed vigorously with 50 μl of SDS (0.1%) and 2 drops of chloroform. This mixed solution was allowed to stand for 5 min at 28°C, mixed well with 200 μl of ONPG (4 mg/ml), and placed in a 37°C water bath. When the yellow color developed (*ca.* 5 min), 1 ml of 1 M Na_2CO_3 was added to stop the reaction. The absorbances of supernatants that were measured after microcentrifuging this solution were related to β-gal activity by the following equation.

$$\text{Activity}=1{,}000\ (A_{420}-1.75\ A_{550})/(\text{tv}\ A_{600})$$

t: time of the reaction in minutes (5 min)
v: volume of the culture used in the assay

E. coli transformants with β-gal activities that dramatically differed from that of the native enzyme were selected as putative mutants. Mutated sequences in β-gal were finally confirmed by using the dideoxy-chain termination method [15] using Sequenase Version 2.0 (Amersham, Buckinghamshire, England) and an ALFexpress automated sequencer (Pharmacia Biotech., Uppsala, Sweden). Therefore, the mutated β-gals and the native enzyme were expressed in *E. coli* and their activities were assayed (Table 3).

Table 3. Relative activities of native type and mutated β-galactosidase transformants (*E. coli* XL^{-1} Blue and *E. coli* AMS66).

	Relative activity (%)	
	E. coli XL^{-1} Blue	*E. coli* AMS66
Native type	100[a]	100[b]
Cys-472-Ser	15.64	14.51
Cys-472-Thr	11.06	10.49
Cys-472-Met	2.76	1.74
His-509-Asn	10.41	8.15
His-509-Phe	2.47	1.6

Actual value of 100[a]: 430, and 100[b]: 400.
E. coli XL^{-1} Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lac*^{q}*Z ΔM15 Tn10*(*Tet*^{r})]} and *E. coli* AMS66 (*recA56 srl::Tn10 ΔlacU169*).

Substituting Cys-472 with Met, and His-509 with Phe, respectively, produced enzymes with *ca.* 1–3% of the activity of the native enzyme. The enzymes Cys-472-Ser, Cys-472-Thr, and His-509-Asn had *ca.* 10–15% of the native enzyme activity. We previously [13, 18] showed that the site-specific mutagenesis of Glu-384, Glu-429, Tyr-475, or Glu-506 results in the loss of >99% of the activity of native β-gal. Thus, these sites were confirmed to be essential for β-gal activity. These results suggest that Glu-384, Glu-429, Tyr-475, and Glu-506 form the primary active site, and that Cys-472 and His-509 are also important but not at the primary active site.

Kinetic Properties: Substrate Effects

The native and the mutated β-gal were prepared by previously described methods [13, 18]. Enzyme concentration was finally corrected to 3.3 mg/ml, and the enzyme was subjected to further enzyme assays. The molecular weight and the protein concentration of the expressed mutated β-gal were exactly the same as those of the native enzyme in SDS-PAGE (data not shown). The activities of native and mutant β-gals were measured using the Miller method [9]. The K_m and V_{max} values of the native and mutant β-gals were determined for each substrate in TES buffer containing 1 mM $MgSO_4$. The substrates used were *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), *p*-nitrophenyl-α-L-arabinopyranoside (PNPA), or *p*-nitrophenyl-β-D-fucopyranoside (PNPF). K_m and V_{max} values were determined by using Hanes-Woolf plots.

Table 4 provides the V_{max}, K_m, and V_{max}/K_m values for the native and mutated enzymes with ONPG, PNPG, PNPA, and PNPF as substrates. Compared with the V_{max} of the native β-gal, the V_{max}'s of the mutated enzymes (Cys-472-Ser, Cys-472-Thr, Cys-472-Met, His-509-Asn, and His-509-Phe) were 60–7,000-fold lower; in particular, the V_{max} values of Cys-472-Met and His-509-Phe mutated enzymes were the lowest. The V_{max} values of the native and mutated β-gals were the highest on ONPG, except for Cys-472-Met and His-509-Phe. V_{max} values of the mutated β-gal on these four substrates decreased in the order ONPG>PNPG≃PNPA>PNPF, whereas V_{max}'s of native β-gal decreased in the order ONPG>PNPG>PNPA>PNPF. These results suggest that in the native β-gal, the ortho position (ONPG) was more efficient than the para form (PNPG). Moreover, the C_6 sugars (galactose, fucose) reacted more efficiently than the C_5 sugar (arabinose), and the hydroxyl C_6 sugars (ONPG, PNPG) more so than the deoxy C_6 sugar (PNPF). V_{max} values for the mutated β-gals on PNPG and PNPA were almost the same. V_{max}'s on PNPF were the lowest for the four substrates. These results suggest that the mutated enzymes cannot distinguish structurally between C_6 (PNPG) and C_5 (PNPA) sugars. These results also imply that a substrate structure containing a C_6 sugar with a hydroxyl

Table 4. Kinetic constants for Cys-472-Ser, Cys-472-Thr, Cys-472-Met, His-509-Asn, His-509-Phe, and native β-galactosidase on ONPG, PNPG, PNPA, or PNPF substrates[a] (V_{max}: $\times 10^{-4}$ mM×ml^{-1}×min^{-1}; K_m: mM).

Strain substrate		Native type	Cys-472-Ser	Cys-472-Thr	Cys-472-Met	His-509-Asn	His-509-Phe
ONPG	V_{max}	5,770	4.6	4.17	0.83	3.47	0.95
	K_m	1.6	0.14	0.53	0.26	0.7	0.1
	V_{max}/K_m	3,606	32.86	7.87	3.19	4.96	9.5
PNPG	V_{max}	644	3.17	2.06	1.48	2.63	1.27
	K_m	0.3	0.27	0.1	0.1	0.23	0.28
	V_{max}/K_m	2,147	11.74	20.6	14.8	11.43	4.54
PNPA	V_{max}	190	3.08	2.42	1.89	2.78	0.26
	K_m	0.2	0.14	0.31	0.14	0.15	0.12
	V_{max}/K_m	950	22	7.81	13.5	18.53	2.17
PNPF	V_{max}	460	0.35	0.29	0.24	1.83	0.09
	K_m	0.15	0.3	0.27	0.23	0.04	0.28
	V_{max}/K_m	3,076	1.17	1.07	1.04	45.75	0.32

[a]V_{max} and K_m values were determined at 25°C in TES assay buffer (30 mM TES, 145 mM NaCl, and 1 mM $MgSO_4$, at pH 7.0).

residue (ONPG, PNPG>>PNPF) is essential for the enzyme-substrate reaction.

In our previous report [18], the V_{max} of mutated β-gal (Tyr-475-Phe, Glu-509-Asp) was the highest on ONPG, and these were similar to the V_{max} of the native enzyme in terms of reactions with the above-mentioned four substrates. This means that enzymes mutated at Tyr-475-Phe or Glu-509-Asp can better distinguish the substrate than Cys-472-Ser, Cys-472-Thr, Cys-472-Met, His-509-Asn, and His-509-Phe. These results are interesting because the four mutated sites (Cys-472, Tyr-475, Glu-506, His-509) are closely located in the same deep binding pocket. The *lacZ* genes from *E. coli* and other β-gals have been sequenced [10, 11, 16], and found to be homologous. The active-site pocket of β-gal from *E. coli* was also found to share extensive homology with various microorganisms, but interestingly, the site of Cys-472 in *L. lactis* 7962 β-gal did not (Table 1).

The large size of the β-gal molecule from *L. lactis* 7962 (996 amino acids, 115 kDa) may account for the fact that the enzyme is very tolerant of changes in its primary sequence, since random alterations in the primary structure of β-gal have such a small effect on enzyme activity. This study shows that catalytic ability of *L. lactis* 7962 β-gal is significantly affected by mutations at Cys-472 or His-509. In this study, we reported the importance of one imidazole group at His-509 and one sulfhydryl group at Cys-472 within the active site of *L. lactis* 7962 β-gal. We conclude that His-509 and Cys-472 are important for the catalytic function of *L. lactis* 7962 β-gal.

β-Gal is known to conduct two catalytic reactions: hydrolysis, in which substrates are broken down at (1→4) glycosidic linkage in the presence of water; or intramolecular galactose transfer, which involves an intramolecular transfer reaction at the (1→6) glycosidic linkage to yields allolactose, the natural inducer of the *lac* operon [3]. We speculated that the enzyme binding site of *L. lactis* 7962 β-gal was intact but its catalytic capabilities (hydrolytic and transgalactosylic) were significantly affected by mutagenesis at His-509 or Cys-472. Further investigations on the steady-state kinetic studies for substrate analogue inhibitors/acceptors will allow us to elucidate distinct reasons for the roles of these two residues and why the enzyme activity was reduced dramatically by mutation.

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