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The Importance of Tyr-475 and Glu-506 in β-Galactosidase from *L. lactis* ssp. *lactis* 7962

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Abstract The secondary and tertiary structures of β-galactosidase from *L. lactis* ssp. *lactis* 7962 were designed using Nnpredict and Sybyl version 6.3. By using site-directed mutagenesis, the mutated enzymes, Tyr-475-Phe and Glu-506-Asp, were generated based on the structural modeling of *L. lactis* ssp. *lactis* 7962. The enzymes Tyr-475-Phe and Glu-506-Asp had <1% of the activity of the native enzyme with ONPG as substrate. The V_{max} values of the mutated enzymes were greatly reduced (1,800~40,000-fold) compared with the value for the native β-galactosidase. However, the K_m values of Tyr-475-Phe and Glu-506-Asp with ONPG, PNPG, PNPF, and PNPA were not significantly different from those of the native enzyme. The results obtained support the suggestion that Tyr-475 and Glu-506 constitute very important parts of the catalytic machinery of the β-galactosidase.

Key words: β-Galactosidase, tyrosine-475, glutamic acid-506, *L. lactis* 7962, substrate binding site

The bioconversion of lactose into lactic acid is an essential process in dairy fermentation. Lactic acid bacteria (LAB) contain phospho-β-galactosidase and β-galactosidase to metabolize lactose [4, 10, 13]. In most *Lactococcus lactis* strains, the predominant enzyme for lactose utilization is phospho-β-galactosidase. However, unlike other lactococci, *L. lactis* ssp. *lactis* 7962 uses β-galactosidase as the major lactose-utilizing enzyme, and in this respect, *L. lactis* 7962 is atypical [3].

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The complete *gal/lac* operon genes of *L. lactis* 7962 have been previously cloned, and genetic studies of the β-galactosidase gene have been determined in our laboratory [2, 7, 8]. The amino acid sequence deduced from the *L. lactis* 7962 β-galactosidase gene (U60828) was compared with those of other microorganisms; namely, β-galactosidase from *E. coli* (V00296), *Klebsiella pneumoniae* (M11441), *Lactobacillus bulgaricus* (M23530), *Streptococcus thermophilus* (M73749), and *Leuconostoc lactis* (M92281), and they were found to exhibit 49.9, 46.1, 30, 27.6, and 22.4% identity, respectively, with the *L. lactis* 7962 protein [11], and *E. coli* enzyme was observed to have the highest identity. Based on the X-ray crystallographic structure of *E. coli* [6], we carried out a structural design on β-galactosidase from *L. lactis* 7962 [11]. By using site-directed mutagenesis, features of the *Lactococcal* 7962 β-galactosidase model have already been predicted; i) negative charges due to Glu-384 and Glu-429 are essential for β-galactosidase activity, and they are identified as Mg^{2+} ligands; ii) the three-dimensional disposition of Mg^{2+} and of its neighboring interactions (Glu-384, Glu-429, Asp-428, and His-386) are very important for the maintenance of β-galactosidase activity [11].

According to the determined amino acid identity, several regions of high similarity were detected. In particular, Tyr-475 and Glu-506 are conserved in a number of β-galactosidases from different sources [1, 5, 14]. It has been suggested that Tyr-475 and Glu-506 are very important residues in *Lactococcal* 7962 β-galactosidase for substrate binding and catalysis. The present study was undertaken to determine whether differential effect in the structure exert on activity, by taking advantage of the site-specific mutagenesis technique. The purpose of this study was to determine if indeed Tyr-475 and Glu-506 residues are of significance.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

The bacterial strains used were *E. coli* XL^{-1} Blue {*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [*F' proAB lac^{q}Z DM*15 *Tn*10 (*Tet^{r}*)]} and *E. coli* AMS66 (*recA*56 *srl::Tn*10 *ΔlacU*169). Strains were stored as glycerol (25%) stocks at −70°C and melted on ice before use. *E. coli* XL^{-1} was used as the recipient in all the transformation experiments and *E. coli* AMS66 was used as the host for enzyme expression. *E. coli* cells were cultured in LB media containing ampicillin 50 μg/ml at 37°C. pBluescript KS^{-} (Stratagene, Ceder Creek, TX, U.S.A.) was used as the vector for all the transformation and mutagenesis experiments.

Modeling of β-Galactosidase (*L. lactis* 7962)

An amino acid homology search was performed using BLAST on the GenBank database, and the amino acid sequence alignments so obtained were analyzed using CLUSTAL V software. Based on the X-ray structure of *E. coli* β-galactosidase [6], the secondary and the tertiary structures of β-galactosidase (*L. lactis* 7962) were predicted using the Nnpredict and Sybyl version 6.3 program (Tripos Inc., St. Louis, MO, U.S.A.).

Mutagenesis

Mutated β-galactosidase was generated using PCR-based site-directed mutagenesis (Exsite PCR-Based Site-Directed Mutagenesis Kit; Stratagene). The procedure used was as described by the manufacturer. Mutagenic reaction conditions were modified slightly according to the primer sequences. The mutagenic primer sequences used in this experiment are listed in Table 1. The *Sal*I and *Pst*I sites were generated at each end of the β-galactosidase gene from *Lactococcus lactis* ssp. *lactis* 7962 by PCR. The PCR-generated β-galactosidase gene fragment was ligated into pBluescript KS^{-} (pBluescript+β-galactosidase) and transformed into *E. coli* hosts. The β-galactosidase so expressed was considered to be the native enzyme and all the mutated β-galactosidases were generated from this native enzyme gene.

Screening of the Mutated β-Galactosidase in *E. coli*

For the screening of mutants, the β-galactosidase activities of *E. coli* transformants harboring mutated β-galactosidase genes were measured according to the modified Miller method [9]. Briefly, an overnight culture of *E. coli* transformant cells in LB medium was inoculated into LB medium containing 2% lactose, and incubated for 15 h at 30°C with shaking. One ml of the cells was harvested by microcentrifugation, washed twice with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 5 mM β-mercaptoethanol, pH 7.0), 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 *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml), and placed in a 37°C water bath. When yellow color developed (*ca.* 5 min), 1 ml of 1 M Na_2Co_3, was added to stop the enzyme-substrate reaction. The absorbance of the supernatants was measured after microcentrifugation of this solution, as described 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.

The *E. coli* transformants, which showed enzyme activities dramatically different from the native enzyme activity, were selected as putative mutants. Mutated sequences in β-galactosidase gene were finally confirmed by the dideoxy-chain termination method [12], using Sequenase Version 2.0 (Amersham, Buckinghamshire, England) and an ALFexpress automated sequencer (Pharmacia Biotech., Uppsala, Sweden).

Preparation of Enzyme

Overnight-cultured *E. coli* cells that harbored the native or the mutated β-galactosidase gene were harvested by centrifugation and washed twice with cold (4°C) deionized water. The washed cells were resuspended in 1/10 vol of TES buffer (30 mM TES, 145 mM NaCl, pH 7.0 at 25°C) and then ultrasonicated for 3 min. After centrifugation at

Table 1. Mutagenic oligonucleotide sequences used in this experiment.

Primer	Sequences	Length (bp)
Tyr-475-Phe	Tyr 5'-TTGTCCGATATGCTAGAGTAG-3' ↓ 5'-TTGTCCGA**TTT**GCTAGAGTAG-3' Phe	23 mer
Glu-506-Asp	Glu 5'-CTAATTCTTTGTGAATATGCTCATG-3' ↓ 5'-CTAATTCTTTGT**GAT**TATGCTCATG-3' Asp	25 mer

12,000 rpm in an A100S-6 (100 ml) rotor (Hanil, Korea) at 4°C for 30 min, the supernatant containing crude cell extract was transferred to a centricon (50 kDa cutoff Amicon, Bedford, MA, U.S.A.). The upper fraction (>MW 50 kDa) so obtained was washed twice with the same buffer. The concentration of the enzyme was then adjusted to 3.3 mg/ml, and the preparation was used for further enzyme assays.

Assays with Substrates

The activities of native and mutant β-galactosidases were measured using the Miller method [9]. The K_m and V_{max} values (25°C) of the native and mutant β-galactosidase were determined for each substrates in TES buffer containing 1 M $MgSO_4$. The substrates used were *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), *p*-nitrophenyl-α-L-arabinopyranoside (PNPA), and *p*-nitrophenyl-β-D-fucopyranoside (PNPF). K_m and V_{max} values were obtained using the Hanes-Woolf plot.

Results and Discussion

Structure and Mutagenesis

Computer modeling of the structure of *Lactococcal* 7962 β-galactosidase at 5 Å level indicated that Tyr-475 (Fig. 1) and Glu-506 (Fig. 2) are positioned in such a way that they could function as an active residue at the substrate binding site. It has been suggested that Tyr-475 may be a catalytically active residue, which has been supported by its conservation in a number of different β-galactosidases [1, 5, 14]. To determine if this was indeed the case, we used the site-directed mutagenesis technique to replace Tyr-475 with a Phe (Tyr-475-Phe), which is very similar to Tyr but does not have a hydroxyl residue (-OH), a supposed acid/base modulator (Fig. 1). The NH_3^+ residue of Tyr-475 interacts (by H-bonding) with the CO^- residue of Glu-506. The three-dimensional structure of residues from Tyr-475 to Glu-506 suggested to contain an active site for substrate binding. To confirm that Glu-506 is important for substrate binding, it was substituted with Asp (Glu-506-Asp). The

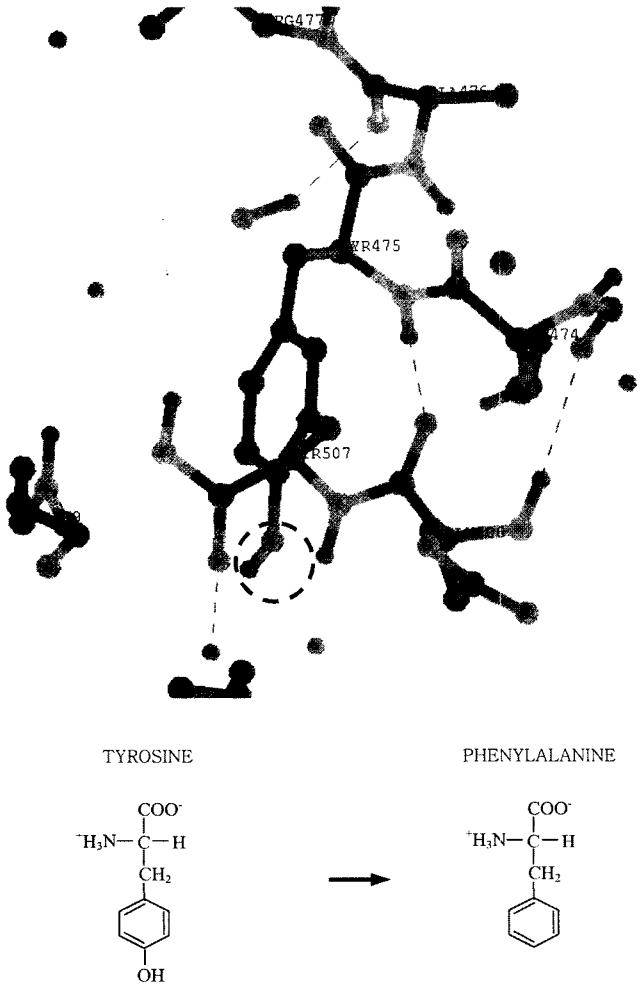

Fig. 1. The proposed active site of β-galactosidase focuses on Tyrosine 475 and its substituted amino acid.

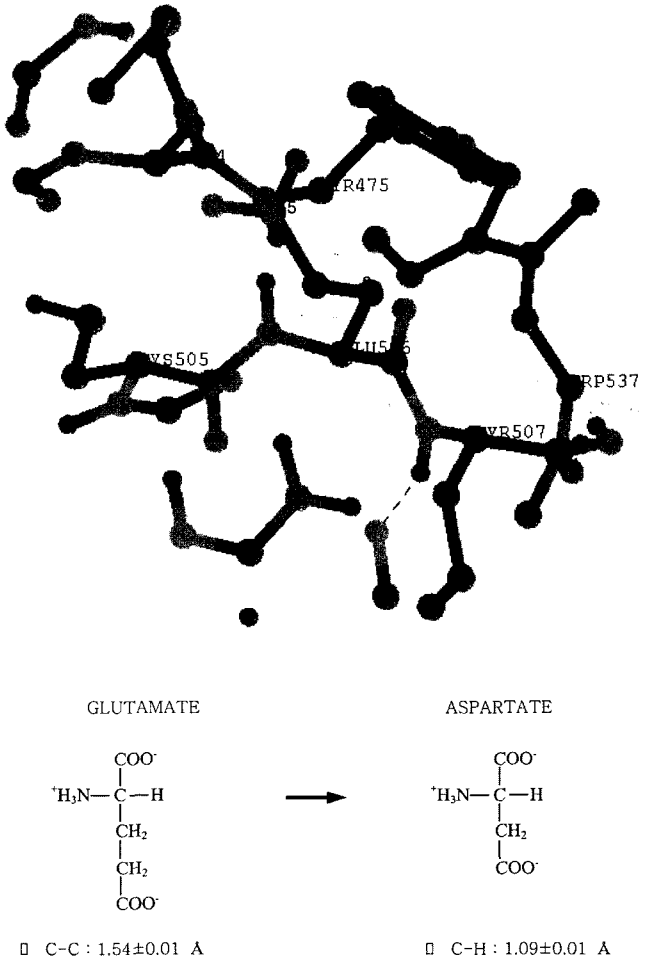

Fig. 2. The proposed active site of β-galactosidase focuses on Glutamic acid 506 and its substituted amino acid.

Table 2. Relative activities of β-galactosidase of the native type and of the mutated 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]
Tyr-475-Phe	0.85	0.7
Glu-506-Asp	1.2	1.1

Actual value of 100[a]: 430 and 100[b]: 400.

Asp-substituted enzyme was constructed, so that the carboxyl group would be retained but that the effective distance to the substrate would be reduced (C-C length: 1.5 Å) (Fig. 2).

Expression of the Mutated β-Galactosidases

The mutated and the native β-galactosidases were expressed in *E. coli*, and their enzymatic activities were assayed. The enzymes of Tyr-475-Phe and Glu-506-Asp appeared to be totally inactive (*ca.* 1% of the activity of the native enzyme) (Table 2). The properties of the resulting enzyme preparation showed that Tyr-475 and Glu-506 were indeed essential for β-galactosidase activity.

Kinetic Properties: Substrate Effects

Table 3 gives the V_{max}, K_m, and V_{max}/K_m values for the native and the mutated enzymes with ONPG, PNPG, PNPA, and PNPF as substrates. ONPG and PNPG have intact C6 hydroxyl methyl groups, whereas PNPA resembles D-galactose but does not have a C6 hydroxyl methyl group. PNPF is also similar to D-galactose, but does not have a C6 hydroxyl group.

Table 3. Kinetic constants for Tyr-475-Phe, Glu-506-Asp, and native β-galactosidase with ONPG, PNPG, PNPA, and PNPF as substrates[a] (V_{max}: $\times 10^{-4}$ mM×ml^{-1}×min^{-1}; K_m: mM).

Strain substrate		Native type	Tyr-475-Phe	Glu-506-Asp
ONPG	V_{max}	5770	2.63	3.13
	K_m	1.6	1.19	0.70
	V_{max}/K_m	3606	2.21	4.47
PNPG	V_{max}	644	0.86	0.88
	K_m	0.3	0.17	0.50
	V_{max}/K_m	2147	5.06	1.76
PNPA	V_{max}	190	0.40	0.14
	K_m	0.2	0.08	0.08
	V_{max}/K_m	950	5.00	1.75
PNPF	V_{max}	460	0.43	0.16
	K_m	0.15	0.15	0.09
	V_{max}/K_m	3076	2.87	1.78

[a]The 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).

V_{max} for the native β-galactosidase with ONPG was the highest among the examined substrates, while the V_{max}'s of the native β-galactosidase with PNPG, PNPF, and PNPA decreased 9-, 12-, and 30-fold, respectively. These results suggested that the ortho orientation was more efficient than para orientation. Moreover, C6 sugar reacted more efficiently than C5 sugar, and hydroxyl C6 sugar more than deoxy C6 sugar as substrates.

Compared with the V_{max} of the native β-galactosidase, the V_{max}'s of the mutated enzymes (Tyr-475-Phe, Glu-506-Asp) were highly reduced. However, the V_{max} for the mutated β-galactosidase with ONPG was the highest, and was similar to the V_{max} of the native enzyme in terms of reaction with the four different substrates. The V_{max} of Tyr-475-Phe mutated enzyme with ONPG as substrate decreased 2,194-fold versus the V_{max} of the native β-galactosidase with ONPG, while the V_{max} reduced to 6,709-fold with PNPG, to 14,425-fold with PNPA, and to 13,419-fold with PNPF. Further consideration revealed that these reductions were due to the loss of a catalytic group (-OH), but were not due to perturbation of the overall enzyme structure. The substitution of a Phe for a Tyr is a very conservative change; structurally, the only difference between the two is the loss of a hydroxyl group. The V_{max} of Glu-506-Asp mutated enzyme with ONPG reduced 1,843-fold, while that of Glu-506-Asp enzyme with PNPG reduced 6,557-fold, with PNPA 41,214-fold, and with PNPF 36,062-fold.

The V_{max} value for the native enzyme with PNPF was found to be higher than that with PNPA. However, the V_{max} values for the mutated β-galactosidases with PNPF and PNPA were almost the same. It seemed that Tyr-475-Phe and Glu-506-Asp could not distinguish difference in structures between the C5-sugar and deoxy C6-sugar substrates. The K_m values of Tyr-475-Phe and Glu-506-Asp enzymes with the four substrates were not found to be dramatically different from those of the native enzyme, demonstrating that the enzyme binding site was essentially intact. A small difference in the K_m values does not necessarily mean that the binding of the substrate is improved in the mutated enzymes, because the equation describing K_m is complex, and involves catalytic rate constants and rate constants for the binding and release of substrate.

In conclusion, this study described a large decrease in catalytic efficiency, when structurally conservative replacements were made at Tyr-475 and Glu-506 of β-galactosidase. It was concluded that Tyr-475 and Glu-506 are most likely very important parts of the catalytic machinery of the enzyme.

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REFERENCES

1. Breunig, K. D., U. Dahlems, S. Das, and C. P. Hollenberg. 1984. Analysis of a eukaryotic β-galactosidase gene: The N-terminal end of the yeast *Kluyveromyces lactis* protein shows homology to the *Escherichia coli lacZ* gene product. *Nucleic Acids Res.* **5:** 2327–2341.
2. Chang, H. C., Y. D. Choi, and H. J. Lee. 1996. Molecular cloning of a β-D-galactosidase gene from *Lactococcus lactis* subsp. *lactis* ATCC7962. *J. Microbiol. Biotechnol.* **6:** 386–390.
3. Crow, V. L. and T. D. Thomas. 1984. Properties of *Streptococcus lactis* strain that ferments lactose slowly. *J. Bacteriol.* **157:** 28–34.
4. De Vos, W. M. and E. E. Vaugham. 1994. Genetics of lactose utilization in lactic acid bacteria. *FEMS Microbiol. Rev.* **15:** 217–237.
5. Fowler, A. V., I. Zabin, M. L. Sinnott, and I. Zabin. 1978. Methionine 500, the site of covalent attachment of an active site-directed reagent of β-galactosidase. *J. Biol. Chem.* **15:** 5283–5285.
6. Jacobson, R. H., X. J. Zhang, R. F. DuBose, and B. W. Mattews. 1994. Three-dimensional structure of β-galactosidase from *E. coli*. *Nature* **369:** 761–766.
7. Lee, J. M., D. K. Chung, J. H. Park, W. K. Lee, H. C. Chang, J. H. Kim, and H. J. Lee. 1997. Cloning and nucleotide sequence of the β-galactosidase gene from *Lactococcus lactis* ssp. *lactis* ATCC7962. *Biotechnol. Lett.* **19:** 179–183.
8. Lee, J. H., J. Y. Choi, J. M. Lee, J. H. Kim, H. C. Chang, D. K. Chung, H. J. Lee. 2000. Expression of the galactosidase mutarose gene from *Lactococcus lactis* ssp. *lactis* ATCC7962 in *Escherichia coli*. *J. Microbiol. Biotechnol.* **10:** 840–843.
9. Miller, J. H. 1972. *Experiments in Molecular Genetics*. CSH NY, pp. 352–355.
10. Park, R. J., K. H. Lee, S. J. Kim, J. Y. Park, S. J. Nam, H. D. Yum, H. J. Lee, H. C. Chang, D. K. Chung, J. H. Lee, Y. H. Park, and J. H. Kim. 2002. Isolation of *Lactococcus lactis* strain with β-galactosidase activity from Kimchi and cloning of *lacZ* gene from the isolated strain. *J. Microbiol. Biotechnol.* **12:** 157–161.
11. Ryoo, H. J., E. J. Yang, H. Y. Chung, H. J. Lee, J. H. Kim, D. K. Chung, J. H. Lee, and H. C. Chang. 2002. Mg^{2+} ligands (Glu-384, Glu-429) of β-galactosidase from *Lactococcus lactis* ssp. *lactis* 7962. *Biotechnol. Lett.* **24:** 691–696.
12. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci.* **74:** 5463–5467.
13. Schroeder, C. J., C. Robert, G. Lenzen, L. L. Mckay, and A. Mercenier. 1991. Analysis of the *lacZ* sequences from two *Streptococcus thermophilus* strains: Comparison with the *Escherichia coli* and *Lactobacillus bulgaricus* β-galactosidase sequences. *J. Gen. Microbiol.* **137:** 369–380.
14. Stockes, H. W., P. W. Betts, and B. G. Hall. 1985. Sequence of the *ebgA* gene of *E. coli*: Comparison with the *lacZ* gene. *Mol. Biol. Evol.* **6:** 469–477.