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Modification of optimal pH in L-arabinose isomerase from *Geobacillus stearothermophilus* for D-galactose isomerization

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Abstract

L-Arabinose isomerase from *Geobacillus stearothermophilus* (GSAI; EC 5.3.1.4) has been genetically evolved to increase the reaction rate toward D-galactose, which is not a natural substrate. To change the optimal pH of GSAI for D-galactose isomerization (pH optimum at 8.5), we investigated the single point mutations influencing the activity based on the sequences of the previously evolved enzymes. Among the seven point mutations found in the evolved enzymes, mutations at Val^{408} and Asn^{475} were determined to be highly influential mutation points for D-galactose isomerization activity. A random mutation was introduced into sites Val^{408} and Asn^{475} (X408V and X475N), and candidates were screened based on non-optimal pH conditions. Among the mutations of X408V and X475N, mutations of Q408V and R408V were selected. The optimal pH of the both mutations Q408V and R408V was shifted to pH 7.5. At the shifted optimal pH, the D-galactose isomerization activities of Q408V and R408V were 60 and 30% higher than that of the wild type at pH 8.5, respectively.

Keywords: Optimal pH shift; L-Arabinose isomerase; *Geobacillus stearothermophilus*; Galactose isomerization; Tagatose

1. Introduction

L-Arabinose isomerase is an enzyme that mediates isomerization between D-galactose and D-tagatose *in vitro* as well as L-arabinose and L-ribulose *in vivo* [1]. The isomerization between D-galactose and D-tagatose using L-arabinose isomerase has recently attracted commercial interest, as D-tagatose is a "Generally Recognized as Safe" (GRAS) low-calorie functional sweetener used as a sugar substitute [2]. L-Arabinose isomerase from several sources has been developed for use in the galactose–tagatose converting enzymatic process [3–6]. The developed L-arabinose isomerases show slower reaction rates for galactose isomerization than for L-arabinose isomerization, and directed evolution is a powerful tool to increase the reaction rate and affinity toward galactose rather than arabinose [7,8].

From the viewpoint of commercial applications, lactose can be a cheap source of galactose to manufacture tagatose. For the tagatose production process, β-galactosidase has been suggested as a lactose hydrolyzing enzyme followed by L-arabinose isomerase as a galactose isomerizing enzyme. Unfortunately, the optimal pH conditions of lactose hydrolysis and galactose isomerization are different; the former is pH 6–7 and the latter is pH 8–8.5. Therefore, industry requires an enzyme with a lower optimum pH for galactose isomerization in order to achieve the two reactions in the same process [9].

Rules for engineering protein activity by rational design are protein-specific, and any such design would require prior detailed structural information. Numerous and intensive site-directed mutagenesis studies have probed this issue. Despite these efforts, no generally applicable rules have been established for rational enzyme design [10,11]. Although protein chemists continue to study the relationships among the sequence, structure, and function of proteins, extensive knowledge is available for only a small fraction of known enzymes. Directed evolution, however, has proven to be useful for modifying enzymes in the absence of such knowledge [12]. It has been used to increase enzyme activity on novel substrates, thermostability, substrate specificity, and enantioselectivity.

Here we report the achievement of lowering the optimal pH of L-arabinose isomerase from *Geobacillus stearothermophilus* (GSAI) for galactose isomerization in response to industrial

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Table 1
Strains and plasmids used in this study

E. coli strain or plasmid	Genotype	Usage/comment	
Strains			
DH5α	*F-Φ80 lacZΔM15 Δ(lacZYA-argF) U169 hsdR17* (r_k-, m_k+) *recA1 endA1 deoR thi-1 supE44 gyrA96 relA1* λ-	*In vivo* mutagenesis, site-directed mutagenesis	CGSC 7855
MC1061	*F-araD139*([]) *Δ(araA-leu)7697 Δ (codB-lacI)3 galK16 galE15 LAM-e14-mcrA0 relA1 rpsL150(strR) spoT1 mcrB9999 hsdR2*	Enzyme production	CGSC 6649
Plasmids			
pLex		Expression vector. P_L promoter, constitutive expression in MC1061, Amp^r	Invitrogen, Carlsbad, CA
pL151		pLex containing GSAI wild type gene	[8]
pL152		Positive clone #1 from directed evolution containing GSAI152	[8]
pL153		Positive clone #2 from directed evolution containing GSAI153	[16]

demand based on the analysis of mutation points from directed evolution.

2. Materials and methods

2.1. Strains, DNA, and plasmids

We used *Escherichia coli* DH5α as the DNA manipulation and plasmid construction strain and *E. coli* strain MC1061 as the enzyme expression strain. L-Arabinose isomerase from *G. stearothermophilus* (*araA*, GenBank access code AF160811) was used for the source of site-directed mutagenesis (SDM) or SDM with degeneracy. All GSAI and its SDM-derived genes were cloned between *Kpn*I and *Eco*RI restriction sites of pLex (Invitrogen, Carlsbad, CA, USA). Plasmid pL151 contained the *GSAI* wild-type gene, while pL152 and pL153 harbored the directed evolved *GSAI* genes (Table 1).

Each mutation from a previous directed evolution study [8] was introduced to be a single point mutation by a modified restriction site PCR method [13]. Plasmid pL151 was used as the template, and the oligonucleotides used in this study are described in Table 2. DNA manipulations were performed using conventional techniques [14]. DNA was recovered from agarose gels with a QIAEX II gel extraction kit (Qiagen, Germantown, MD, USA). Oligonucleotides were synthesized in the oligonucleotide synthesis facility of Bioneer Co. (Taejon, Korea), and DNA sequencing was performed in the DNA sequencing facility of Macrogen Co. (Seoul, Korea).

2.2. Enzyme activity analysis and optimum pH-shifted mutant screening

To analyze relative enzyme activity, the *E. coli* MC1061 cells harboring *GSAI mutant* genes in a plasmid were used for the source of enzyme. Actively growing cells (O.D. = 1) in the LB-medium with ampicillin (50 μg/ml) were harvested and washed twice by centrifuge. The harvested cells were resuspended in a 50 mM Tris–HCl buffer (pH 8.5) and disrupted by sonication (Sonics & Materials, Inc., Danbury, CT, USA) at 4 W for 1 min in an ice-bath. After removal of cell debris by centrifuge, the crude extract was precipitated and dialyzed as previously described [3]. The partial purified enzyme solutions were used for further experiments. To determine the specific activity of mutated GSAI, a reaction mixture (1 ml) containing 50 μmol galactose and 100 μl crude extract in 50 mM Tris–HCl (pH 8.5) was incubated at 60 °C for 2 h. In the experiment for optimal

Table 2
Oligonucleotides used in this study

Oligonucleotides	Usage
GCCCTGAAGAAGGGCTTTATTTGA	For nascent N-terminal GSAI
TTAACCAATGATGCTGTCATTACGTCC	For nascent N-terminal GSAI with the restriction site removal
CCCTGTACGATTACTGCAGGTGC	For nascent C-terminal GSAI
CATCGAGCAACTCGTCCACTTTTTGTTCAG	Mutagenesis for D228N
CTTTGCCATCGGCCACCACTTTCATCAAC	Mutagenesis for V322M
CGCGCCTCGCCGTCGTCAAACACGAG	Mutagenesis for D384G
CTAAATCGATCAGCGTAGCATTGACCGCC	Mutagenesis for T393S
GTTTCACCGCATCGGCTTCATTGACAATGAG	Mutagenesis for A408V
GCGACGGGCGCGGGTTCCATAAAATGCG	Mutagenesis for N428K
CGGACGTATGTTCCTTGATCACGACGCAT	Mutagenesis for K475N
GACGGACGTATGTTCCTGGATCACGACGCATTC	Mutagenesis for Q475N
GACGGACGTATGTTCACGGATCACGACGCATTC	Mutagenesis for R475N
GGTTTCACCGCATCGCCTTCATTGACAATGAGAC	Mutagenesis for G408V
GGTTTCACCGCATCCTTTTCATTGACAATGAGAC	Mutagenesis for A408V
GACGGACGTATGTTCNNNGATCACGACGCATTC	Mutagenesis for X475N
GGTTTCACCGCATCNNNTTCATTGACAATGAGAC	Mutagenesis for X408V

Underline indicates the mismatched base. N indicates the any of four nucleotides. X indicates the any of 20 amino acids.

pH determination, the pH of the Tris–HCl buffer was varied in the range pH 7.0–9.0. Tagatose, the isomerization product, was analyzed by the cystein–carbazole method using a spectrophotometer at 570 nm [15]. One unit was defined as 1 nmol tagatose formation in 1 min. The protein concentration was assayed using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as a standard.

For the screening of the optimum pH-modified mutation, enzyme activities derived from *E. coli* MC1061 cells harboring GSAI X408V or GSAI X475K (where X is any 20 amino acids) in a plasmid were screened in a pH 7.5 buffer as follows. Colonies grown on LB-agar medium containing ampicillin (50 μg/ml) were transferred to 96-well plates containing 50 μl LB medium with 50 μg/ml ampicillin. Cells in the 96-well plates were incubated at 37 °C for 1 h to allow galactose isomerase expression. After adding 50 μl galactose solution (10 g/l in 50 mM Tris–HCl, pH 7.5), cell density was estimated by O.D. at 570 nm with the aid of an ELISA reader (Microplate Reader 550, Bio-Rad Laboratories). Enzyme activity was measured at 570 nm after incubation of the 96-well plate at 60 °C for 2 h. Colonies with greater specific activity than the colonies containing pL151 at pH 7.5 were selected.

3. Results

3.1. Effect of single point mutations from the directed evolution

The GSAI152 and GSAI153 were evolved from GSAI to increase the reaction rate for galactose isomerization in place of arabinose, the original substrate. Five amino acid mutations of GSAI (GSAI152: D228N, D384G, T393S, N428K, and K475N) enhanced galactose isomerization activity by 11-fold over the wild-type enzyme, and three amino acid mutations of GSAI (GSAI153: V322M, T393S, and A408V) showed a 17-fold increase [8,16]. The single amino acid mutations on GSAI were introduced by SDM based on the sequence of the directed evolved enzymes, and the most influencing amino acid mutation in galactose isomerization was investigated (Table 3). The activity of GSAI D228N decreased drastically. The mutation at D384G showed little effect on the activity, while GSAI V322M, T393S, A408V, N428K, and K475N showed 2- to 11-fold greater activity than the wild type. The A408V and K475N were the most influential mutations on the galactose isomerization activity among those tested and were selected as sites for further investigations into pH shifts.

3.2. Effect of mutations at Val^{408} and Asn^{475} on the pH optimum

Val^{408} or Asn^{475} was replaced with a random amino acid. The plasmids containing GSAI X408V and GSAI X475N generated by SDM with degenerate PCR were transformed into cells, and mutations were screened based on the higher galactose isomerization activity than the GSAI wild type at pH 7.5, which is not the optimal condition. Among the 100 colonies tested, two mutant colonies were screened in the test and characterized as Q408V and R408V following the results of nucleotide sequencing. After partial purification of GSAI Gln^{408} and GSAI Arg^{408}, the relative activity depending on pH was analyzed (Fig. 1). The optimal pH of GSAI wild type was shifted from pH 8.5 to 7.5 by the mutations GSAI Gln^{408} and GSAI Arg^{408}. The galactose isomerization activities of GSAI Gln^{408} and GSAI Arg^{408} at pH 7.5 were 60 and 30% higher than that of the wild type at pH 8.5, respectively.

Table 3
Galactose isomerization activity of GSAI and its mutants

Mutation	Specific activity for D-galactose isomerization (U/mg-protein)	Description
GSAI wild type	43 (±12)	Wild type (GenBank AF160811)
GSAI152	490 (±35)	Five mutations: D228N, D384G, T393S, N428K, K475N
GSAI153	740 (±52)	Three mutations: V322M, T393S, A408V
GSAI D228N	4 (±2)	Single mutation
GSAI V322M	270 (±17)	Single mutation
GSAI D384G	56 (±10)	Single mutation
GSAI T393S	110 (±16)	Single mutation
GSAI A408V	480 (±38)	Single mutation
GSAI N428K	260 (±14)	Single mutation
GSAI K475N	390 (±41)	Single mutation

Unit: 1 nmol tagatose formation per min at pH 8.5. Values in parentheses represent the standard deviation of triplicated experiments.

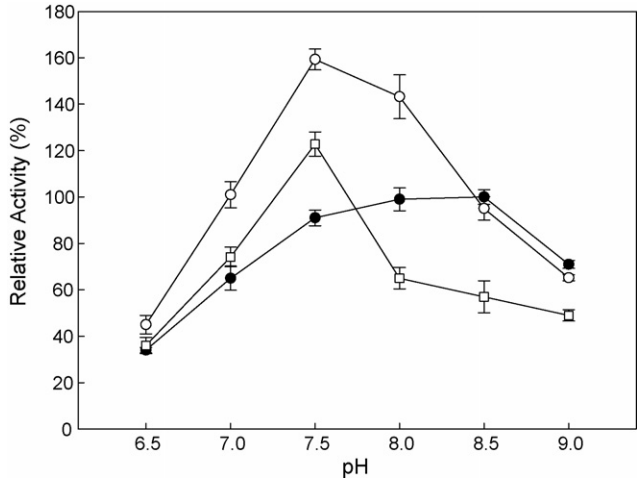

Fig. 1. Effect of single amino acid mutation on the optimal pH of GSAI. Symbols represents: wild type GSAI (●), GSAI Gln^{408} (○), GSAI Arg^{408} (□). Relative activity was calculated based on the specific activity of GSAI at pH 8.5 as 100%. Error bar represents the standard deviation of triplicated experiments.

4. Discussion

We have previously reported on the directed evolution of GSAI for galactose isomerization. The mutation points in the first round of directed evolution were investigated to understand galactose isomerization of GSAI and the possibility of further evolution. Based on the results of single amino acid mutation

Table 4
Mutation effect of 408 and 475-site on the galactose isomerization activity

Mutation	Specific activity for D-galactose isomerization (U/mg-protein)	Description
GSAI Val^{408}	43 (±12)	Wild type
GSAI Ala^{408}	480 (±38)	Found in GSAI153
GSAI Gly^{408}	370 (±42)	Smaller neutral residue
GSAI Leu^{408}	50 (±14)	Bulkier neutral residue
GSAI Gln^{408}	62 (±19)	Polar residue, optimal pH shifted
GSAI Arg^{408}	29 (±5)	Positive charged residue, optimal pH shifted
GSAI Asn^{475}	43 (±12)	Wild type
GSAI Lys^{475}	390 (±41)	Found in GSAI152
GSAI Gln^{475}	370 (±44)	Polar residue
GSAI Arg^{475}	380 (±52)	Positive charged residue
GSAI Ala^{408}	480 (±38)	Found in GSAI153
GSAI Lys^{475}	390 (±41)	Found in GSAI152
GSAI Ala^{408} Lys^{475}	590 (±36)	Found in GSAI152 or GSAI153

Unit: 1 nmol tagatose formation per min at pH 8.5. Values in parentheses represent the standard deviation of triplicated experiments.

GSAIs, mutations at Val^{408} and Asn^{475} were found to be the most influential mutation points in the galactose isomerization activity. It was surprising that little activity change was detected in mutation T393S, because this mutation was found in both GSAI152 and GSAI153.

Table 4 summarizes the mutational study at sites 408 and 475 of GSAI. In the analysis of the mutation at site 408, the enzyme activity increased 11-fold by replacing Val^{408} with Ala^{408}. When Ala^{408} was replaced with the bulkier neutral amino acid Leu^{408}, the activity dropped to 10% of GSAI A408V. By replacing Ala^{408} with Gly^{408}, the smallest amino acid, the activity was decreased to 68% of GSAI Ala^{408}. The amino acid at 408 was considered to require a certain size residue to show activity for galactose isomerization. In the case of the mutation on site 475, we detected a 9-fold increase by replacing Asn^{475} with Lys^{475}. As long as the amino acid at 475 was a positively charged residue (Gln^{475} and Arg^{475}), the galactose isomerization activity was the same as that of GSAI K475N. When Val^{408} and Asn^{475} were introduced at the same time, the activity increased to 14-fold that of the wild type. The specific activity of the GSAI A408V K475N double mutation showed higher activity (590 ± 36 U/mg-protein) than that of the each single mutation of GSAI A408V (480 ± 38 U/mg-protein) or GSAI K475N (390 ± 41 U/mg-protein), and less than that of GSAI153 (GSAI V322M T393S A408V triple mutation, 740 ± 52 U/mg-protein). Mutations besides those at sites 408 and 475 seemed to affect the enzyme structure to make it more suitable for galactose isomerization than each single mutation alone.

Although we do not fully understand the entire enzyme structure–activity relationship, we were able to predict that the amino acids at sites 408 and 475 would influence the galactose isomerization more than any other sites. Therefore, mutations at 408 and 475 should frequently provoke modification of galactose isomerization activity, and further, may have a greater chance of improving the enzyme. Structural information of GSAI would explain the relationship between the amino acid at 408 or 475 and galactose isomerization activity; further investigations are currently under way.

Based on SDM with degeneracy, we found mutations that changed their optimal pH at site 408. The neutral amino acids of Val^{408} (in the wild type) or Ala^{408} (in GSAI153) were changed to Gln^{408} or Arg^{408}, which are polar or positively charged amino acids. The difference in the electric field on site 408 may have influenced the microenvironment of the active site, which allowed galactose to be isomerized at lower pH. A plausible explanation may be that the positively charged or polar side group near the active site repelled positive protons and made the microenvironment more alkaline than the outside. Pyun and co-workers lately reported that mutation of positively charged lysine near active site into glutamate shifted pH optimum 6 to 7 in the *Alicyclobacillus acidocaldarius* AI (AAAI) whereas mutation of negatively charged glutamate near active site into lysine shifted pH optimum 8 to 7 in the *Bacillus halodurans* AI (BHAI) [17]. The above results are good agreement with the positively charged or polar side group near active site is responsible for the optimal pH change. Another consideration may be the change of divalent cation-binding properties. The GSAI can be activated by divalent cations, which implies that GSAI isomerizes via a hydride-shift mechanism using metal cofactors such as xylose isomerase [18]. As well as the metal effect on catalytic activity, Lee et al. [19] suggested metal ion is necessary for proper protein folding. A change of neutral amino acid into a polar or a positively charged one near active site may influence the metal location resulted in the optimal pH change. The change in pH profile may reflect an underlying change in cation-binding properties via mutations near active sites.

Bulkier Arg^{408} made the enzyme less active than Gln^{408} (Fig. 1), and this result agreed with the results of the analysis of site 408 (the bulkier Leu^{408} rendered the enzyme less active than Ala^{408}; Table 4).

From a commercial viewpoint, the two-step enzyme process for tagatose production from lactose (β-galactosidase for lactose hydrolysis and L-arabinose isomerase for galactose isomerization) has been restrictive because of the differences in optimal pH. Introducing a positively charged or polar amino acid at site 408 to another commercially evolved GSAI may modify the pH operation conditions. Since no structural information is available for L-arabinose isomerase, SDM or SDM with degeneracy at the possible site after directed evolution would be an expedient way to develop a commercial enzyme.

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