Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 43 (2006) 108–112
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

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

Deok Kun Oh [a], Hyo Jung Oh [b,1], Hye Jung Kim [a], Jina Cheon [b], Pil Kim [b,*]

[a] *Department of Molecular Biotechnology, Konkuk University, Seoul 143-701, South Korea*
[b] *Department of Biotechnology, The Catholic University of Korea, Bucheon 420-743, South Korea*

Available online 31 July 2006

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

* Corresponding author. Tel.: +82 2 2164 4922; fax: +82 2 2164 4865.
E-mail addresses: hyojeong@snu.ac.kr (H.J. Oh), kimp@catholic.ac.kr (P. Kim).
[1] Present address: Bio-MAX Institute, Seoul National University, Seoul, South Korea. Tel.: +82 2 887 2693; fax: +82 2 887 2692.

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.06.015

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.