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Increase in D-tagatose production rate by site-directed mutagenesis of L-arabinose isomerase from *Geobacillus thermodenitrificans*

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Abstract

Among single-site mutations of L-arabinose isomerase derived from *Geobacillus thermodenitrificans*, two mutants were produced having the lowest and highest activities of D-tagatose production. Site-directed mutagenesis at these sites showed that the aromatic ring at amino acid 164 and the size of amino acid 475 were important for D-tagatose production. Among double-site mutations, one mutant converted D-galactose into D-tagatose with a yield of 58% whereas the wild type gave 46% D-tagatose conversion after 300 min at 65 °C.

Introduction

L-arabinose isomerase (EC 5.3.1.4) catalyzes the conversion of both D-galactose to D-tagatose and L-arabinose to L-ribulose, owing to the similar configurations of the substrates (Cheetham & Wootton 1993). The sweetness and taste quality of D-tagatose are similar to those of sucrose, but D-tagatose is non-calorific (Levin 2002). Because of these properties, D-tagatose is of interest as a functional sweetener. Although many L-arabinose isomerases have been used for D-tagatose production (Roh *et al.* 2000, Kim *et al.* 2002, 2003a, b, Jørgensen *et al.* 2004, Lee *et al.* 2004, Kim & Oh 2005), their reaction rates are too slow for industrial processes. Directed evolution of the L-arabinose isomerase gene has been suggested as a powerful tool for increasing the reaction rate (Kim *et al.* 2001).

In this study, we have investigated the increase of D-tagatose production by site-directed mutagenesis of L-arabinose isomerase obtained from *Geobacillus thermodenitrificans*, based on our analysis of mutation sites obtained from multiple random mutageneses.

Materials and methods

Microorganisms, plasmid and medium

Escherichia coli strain BL21(DE3) was used as the host for pET-GTAI, an expression vector derived from pET15b (Novagen, Madison, WI, USA) and the L-arabinose isomerase gene of *Geobacillus thermodenitrificans* (GenBank access code AY302754; Baek *et al.* 2004, Kim & Oh 2005). The L-arabinose isomerase gene and all genes derived from its site-directed mutagenesis were cloned between the *Nde*I and *Bam*HI restriction sites. The transformed *E. coli* BL21(DE3) was cultivated at 37 °C for 5 h in a 250 ml flask containing 50 ml Luria-Bertani (LB) medium.

Random and site-directed mutagenesis of L-arabinose isomerase

Random mutagenesis of L-arabinose isomerase was performed using an error-prone PCR with a PCR mutagenesis kit (ClonTech Laboratories, Palo Alto, CA, USA). Each mutation site obtained from the

random mutagenesis was introduced as a single- or double-site mutation by site-directed mutagenesis based on site-specific mutagenesis by overlap extension (Aiyar *et al.* 1996). Oligonucleotides were synthesized by the oligonucleotide synthesis facility of Bioneer Co. (Daejon, Korea), and DNA sequencing was performed by the DNA sequencing facility of Macrogen Co. (Seoul, Korea).

Enzyme purification and determination of kinetic parameters

Cells were harvested from culture broth by centrifugation at 15 000 × *g* for 20 min at 4 °C, washed, and then resuspended in 50 mM Tris/HCl buffer (pH 8.0). The resuspended cells were disrupted by passing them twice through a French press at 1000 atm. Enzymes from the disrupted cells were purified by heat treatment, HisTrap HP chromatography, and Resource Q anion chromatography (Amersham Biosciences, Uppsala, Sweden). The apparent K_m (mM) and k_{cat} (min^{-1}) for each substrate were calculated by standard methods.

Analytical methods

The activity of L-arabinose isomerase was determined by measuring D-tagatose or L-ribulose formation using D-galactose or L-arabinose as a substrate. The reaction was performed with 100 mM D-galactose or L-arabinose at 65 °C in 50 mM Tris/HCl buffer (pH 8.5) for 30 min. D-Tagatose or L-ribulose were determined by a Bio-LC with an electrochemical detector (Dionex ED-50, Sunnyvale, CA, USA) using a Dionex Carbo PAC MA1 column with 480 mM NaOH at 0.4 ml min^{-1}.

Results and discussion

Effect of single-site mutations on the isomerization activity of L-arabinose isomerase

To investigate D-tagatose production (D-galactose isomerization), multiple random mutageneses were performed on wild type L-arabinose isomerase obtained from *G. thermodenitrificans.* Among the 497 amino acids comprising the enzyme, 10 amino acid sites substantially affected D-tagatose production. Single-site mutations for each of the 10 amino acids were derived by site-directed mutagenesis based on our analysis of mutation sites obtained from multiple random mutageneses (Table 1). In four mutants, D-galactose isomerization activities decreased, but increased in six mutants. When the isomerization activity of D-galactose increased, the K_m for D-galactose decreased. In general, the isomerization activity of L-arabinose increased with the increasing activity of D-galactose. W164G mutant had the lowest D-tagatose production and N475K mutant had the highest.

We investigated amino acid sites 164 (Try) and 475 (Asn) further for isomerization activity and substrate affinity because of their influence on D-tagatose production (Table 2). When the aromatic ring of amino acid 164 was removed, isomerization activity and affinity for D-galactose decreased substantially. The replacement of the aromatic ring with a histidine ring resulted in greatly decreased isomerization activity and a slight increase in D-galactose affinity, suggesting that the aromatic ring of amino acid 164 of L-arabinose isomerase may be an active site for D-galactose isomerization. Among several amino acids with a positively charged residue that were substituted at amino acid 475, lysine was of suitable size and showed the highest activity for the D-galactose. The substitution of asparagine and glutamine, which are

Table 1. Isomerization activities and substrate affinities of wild-type and mutant L-arabinose isomerases from *Geobacillus thermodenitrificans.*

Organism	Enzyme activity (%)*		K_m (mM)	
	D-galactose	L-arabinose	D-galactose	L-arabinose
Wild type	100	100	144	97
W164G	2	16	1105	36
N228D	34	44	161	123
K320R	234	149	148	56
M322V	90	111	280	15
G384D	18	27	713	163
S393T	118	70	201	188
V408A	203	230	97	76
K428N	249	183	90	59
C450S	300	200	67	51
N475K	302	225	45	69

*100% activity was 0.14 U/mg for D-galactose and 4.82 U/mg for L-arabinose.

Table 2. Effect of single-site mutation on isomerization activity and substrate affinity.

Enzyme	R group	Enzyme activity (%)*		K_m (mM)	
		D-galactose	L-arabinose	D-galactose	L-arabinose
Trp[164]	Aromatic ring	100	100	144	97
Phe[164]	Aromatic ring	122	98	101	54
His[164]	Positively charged ring	8	12	130	90
Gly[164]	H	2	16	1105	36
Asn[475]	2C 1N	100	100	144	97
Gln[475]	3C 1N	231	139	105	94
Lys[475]	4C 1N	302	170	45	69
Arg[475]	4C 3N	201	184	173	82

*100% activities as given in Table 1.

smaller than lysine, and arginine, which is larger, decreased D-tagatose production. Therefore, we selected N475K as a single-site mutant.

Effect of double-site mutations on the isomerization activity of L-arabinose isomerase

We engineered double-site mutations of K320R, V408A, K428N, or C450S with N475K, because the D-tagatose production activity of these mutants was more than two-fold higher than that of the wild type enzyme (Table 3). D-Tagatose production was lower than that of the single-site mutant (N475K) for the K320R-N475K and V408A-N475K double-site mutants, but higher for the K428N-N475K and C450S-N475K double-site mutants. Among the double-site mutants, the C450S-N475K mutant had the highest D-tagatose production and was selected as a double-site mutant.

Kinetic parameters and D-tagatose production of wild-type, single-site mutant, and double-site mutant L-arabinose isomerases

The kinetic parameters of the wild-type, single-site mutant, and double-site mutant L-arabinose isomerases from *G. thermodenitrificans* using D-galactose and L-arabinose as substrates are presented in Table 4. The turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) using D-galactose and L-arabinose increased in order from the wild type to the single-site mutant to the double-site mutant enzymes. For D-galactose and L-arabinose, the k_{cat}/K_m of the double-site mutant enzyme was 6.2 and 2.8 times higher, respectively, than the values for the wild-type enzyme. For L-arabinose, the k_{cat}/K_m of the wild type and double-site mutant was 96 and 44 times higher, respectively, than the values obtained using D-galactose. These results indicate that the L-arabinose isomerase was more

Table 3. Effect of double-site mutations on isomerization activity and substrate affinity.

Enzyme	Enzyme activity (%)*		K_m (mM)	
	D-galactose	L-arabinose	D-galactose	L-arabinose
Wild type	100	100	144	97
N475K	302	170	45	69
K320R, N475K	297	213	103	58
V408A, N475K	272	176	257	66
K428N, N475K	316	243	149	168
C450S, N475K	407	259	75	71

*100% activities as given in Table 1.

Table 4. Kinetic parameters of wild-type and mutant L-arabinose isomerases for D-galactose and L-arabinose.

Enzyme	D-galactose			L-arabinose		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Wild type	408	204	0.5	142	6816	48
N475K	290	735	2.5	100	13083	131
C450S, N475K	339	1046	3.1	106	14434	136

specific for L-arabinose than for D-galactose and that the isomerization activity of the double-site mutant increased more specifically for D-galactose than for L-arabinose.

The D-tagatose formation profiles using the L-arabinose isomerases are shown in Figure 1. The double-site mutant of L-arabinose isomerase had the highest D-tagatose production rate, followed by the single-site mutant and wild-type enzymes. After 400 min, the D-tagatose yield from D-galactose was 46% for the wild type, 55% for the single-site mutant, and 58% for the double-site mutant enzyme.

In conclusion, a double-site mutant, C450S-N475K, of L-arabinose isomerase was obtained by site-directed mutagenesis which had 20% higher D-tagatose conversion than the wild type enzyme. This mutant enzyme may be valuable for commercial production of D-tagatose.

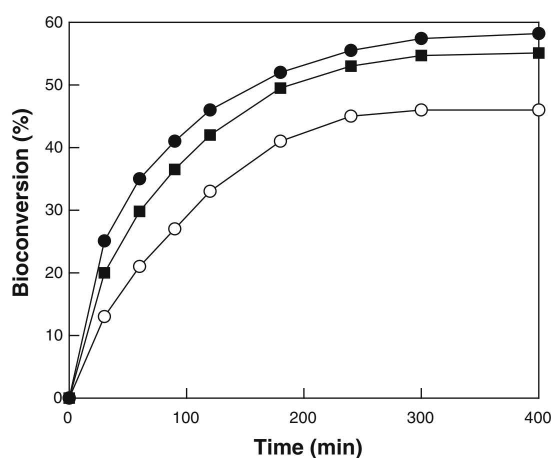

Fig. 1. Time course of D-tagatose production from D-galactose by the wild-type (○), N475K single-site mutant (■), and C450S-N475K double-site mutant (●) L-arabinose isomerases. The bioconversion of D-galactose into D-tagatose by the enzyme was carried out at 65 °C in 50 mM EPPS buffer solution (pH 8.5) containing 10 mM galactose.

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