

RESEARCH ARTICLE

Biochemical properties of L-arabinose isomerase from *Clostridium hylemonae* to produce D-tagatose as a functional sweetener

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Citation: Nguyen T-K, Hong M-G, Chang P-S, Lee B-H, Yoo S-H (2018) Biochemical properties of L-arabinose isomerase from *Clostridium hylemonae* to produce D-tagatose as a functional sweetener. PLoS ONE 13(4): e0196099. <https://doi.org/10.1371/journal.pone.0196099>

Editor: Ligia O Martins, Universidade Nova de Lisboa, PORTUGAL

Received: December 2, 2017

Accepted: April 8, 2018

Published: April 23, 2018

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work received support from the High Value-added Food Technology Development Program (Project number: 115035-03-2-HD020), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea to SHY. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

D-Tagatose has gained substantial interest due to its potential functionalities as a sucrose substitute. In this study, the gene *araA*, encoding L-arabinose isomerase (L-AI) from *Clostridium hylemonae* (DSM 15053), was cloned and expressed in *Escherichia coli* BL21 (DE3). This gene consists of 1,506 nucleotides and encodes a protein of 501 amino acid residues with a calculated molecular mass of 56,554 Da. Since L-AI was expressed as an intracellular inclusion body, this enzyme was solubilized with guanidine hydrochloride, refolded, and activated with a descending concentration gradient of urea. The purified enzyme exhibited the greatest activity at 50°C, pH 7–7.5, and required 1 mM of Mg²⁺ as a cofactor. Notably, the catalytic efficiency (3.69 mM⁻¹sec⁻¹) of L-AI from *C. hylemonae* on galactose was significantly greater than that of other previously reported enzymes. The bioconversion yield of D-tagatose using the *C. hylemonae* L-arabinose isomerase at 60°C reached approximately 46% from 10 mM of D-galactose after 2 h. From these results, it is suggested that the L-arabinose isomerase from *C. hylemonae* could be utilized as a potential enzyme for D-tagatose production due to its high conversion yield at an industrially competitive temperature.

Introduction

D-Tagatose, a rare natural sweetener, is an isomer of aldohexose D-galactose and a C-4'-epimer (stereoisomer) of D-fructose. The taste of tagatose is closely equivalent to that of sucrose with no cooling effect or aftertaste, and has a relative sweetness value of 92% when compared to that of 10% solutions. This sugar has a significantly lower caloric value of 1.5 kcal/g, and the consumption of tagatose results in a reduced energy intake as well as promotion of weight loss at medically desirable rates [1, 2], which has also been used in the treatment of obesity [3, 4]. Owing to its lack of a glycemic effect [5], D-tagatose can be safely consumed by diabetic

Competing interests: The authors have declared that no competing interests exist.

patients [6]. Several intensive studies have been conducted to investigate the different aspects of D-tagatose in food and pharmaceutical formulations, such as the stability of D-tagatose in food and drinks [7, 8], clinical trials for pharmaceutical applications [9], and consumer evaluations [10].

The production of D-tagatose from D-galactose can be achieved through chemical and enzymatic methods. However, the chemical method is limited for industrial applications because of the use of calcium as a catalyst, complicated processes, and the formation of chemical waste and unexpected by-products [11]. The production of D-tagatose by enzymatic methods is regarded as an environmentally friendly protocol, and has thus been studied more intensively in recent years. L-Arabinose isomerase (L-AI; EC 5.3.1.4) is the most effective biocatalyst for the isomerization of D-galactose to D-tagatose, which is an intracellular enzyme catalyzing the reversible isomerization of L-arabinose to L-ribulose and D-galactose to D-tagatose [12]. L-AIs originating from various microorganisms have been identified and biochemically characterized, including *Escherichia coli* [13, 14], *Lactobacillus plantarum* [15], *Bacillus subtilis* [16], *Lactobacillus reuteri* [17], *Bacillus licheniformis* [18], *Geobacillus thermodenitrificans* [11], *Thermoanaerobacterium saccharolyticum* [19], and *Thermotoga maritima* [20]. The ideal enzyme for production of D-tagatose in the food industry requires high thermostability and a weakly acidic pH optimum [5, 21]. The L-AI reaction at high temperature shifts the bioconversion equilibrium to D-tagatose production with an increasing reaction rate between D-galactose and D-tagatose, thereby decreasing the viscosity of the reaction mixtures and reducing the degree of microbial contamination [19, 22]. However, the thermophilic and hyperthermophilic L-AIs from various sources mainly present a slightly alkaline pH optimal range around 7–8.5 [18, 23], and show low activity at acidic pH conditions [24]. The mesophilic and thermophilic L-AIs also require Mn^{2+} and/or Co^{2+} as cofactors to enhance their isomerization activity and to maintain thermostability [23]. Moreover, ion-independent and Mn^{2+} -dependent L-AIs are more favorable for the food industry owing to the toxicity of Co^{2+} [20, 23].

The objective of this study was to investigate a novel L-AI from *Clostridium hylemonae* DSM 1505, and its potential application in the environmentally friendly production of D-tagatose. The gene *araA* encoding L-AI from *C. hylemonae* DSM 15053 was cloned and expressed in *Escherichia coli* BL21 (DE3). The recombinant enzyme was purified and its biochemical characteristics were determined to optimize the production of D-tagatose from D-galactose by L-AI, as a highly valuable sweetener.

Materials and methods

Bacterial strains, plasmid, and chemicals

C. hylemonae (DSM 15053) was employed as a source of L-AI. *E. coli* JM 109 [*F'* *traD36 proA⁺B⁺ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14 gyrA96 recA1 relA1 endA1 thi hsdR17*] (Promega, Madison, WI, USA) was applied as a host cell for gene cloning and DNA manipulation. *E. coli* BL21 (DE3) strain [*F' ompT hsdS_B (r_B⁻ mB⁺) gal dcm (DE3)*] (Novogen, Darmstadt, Germany) was applied as a host cell for expressing the enzyme. pGEM-T (Promega) and pET-28a(+) (Novogen, Darmstadt, Germany) vectors were employed as cloning and expression vectors, respectively. T4 DNA ligase was purchased from Elpis Biotech, Inc. (Daejeon, Korea). D-Tagatose and D-galactose of the highest purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used in this study were of reagent grade.

Cloning of the *araA* gene from *C. hylemonae*

C. hylemonae (DSM 15053) was cultured in brain heart infusion broth (BD, Sparks, MD, USA) under anaerobic conditions (Hungate tubes; N_2) at 37°C for 24 h. The harvest was used

directly as a template DNA for polymerase chain reaction (PCR). The full-length nucleotide sequence of the *araA* gene was obtained with two oligonucleotides designed as PCR primers, 5'-GAGACAGGATCCATGATAAAAAAGCAAAGAA-3' (Forward) and 5'-ACATTCCTCGAGCTAAATCCCCAGCTTATAGGC-' (Reverse), containing restriction sites of *Bam*HI and *Xho*I (underlined). The PCR was performed by iQ™5 Multicolor Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA), and the amplified DNA fragment (1.5 kb) was ligated into the pGEM-T vector. The obtained *araA* sequence was compared with the original *araA* sequence in the National Center for Biotechnology Information (NCBI) database. The target gene, L-AI from *C. hylemonae* (*ChAI*), was then subcloned into the expression vector pET-28a(+) with the *Nde*I and *Hind*III restriction sites. The sub-cloned pET28a-*ChAI* was transformed into *E. coli* BL21 (DE3) for protein expression.

Refolding and purification of the recombinant L-AI

After isopropyl- β -D-thiogalactoside induction at 16°C for 20 h, the cells were collected by centrifugation at 5,500 $\times g$ at 4°C for 10 min, and the precipitant was resuspended in 20 mM Tris-HCl (pH 8.0). The suspended cells were disrupted by the Vibra™ Cell VC 750 disruptor (Sonic & Materials, Inc., Newtown, CT). The pellet harboring the inclusion body of L-AI was dispersed in a resuspending buffer [2 M urea, 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2% Triton X-100] to remove cell membrane proteins. After centrifugation, the pellet of recombinant L-AI as an inclusion body was solubilized in a binding buffer (pH 8.0) consisting of 20 mM Tris-HCl, 0.5 M NaCl, 6 M guanidine hydrochloride, and 2 mM β -mercaptoethanol. The dissolved inclusion body was collected by centrifugation at 13,000 $\times g$ at 4°C for 20 min, and the purification of 6 \times His-tagged recombinant L-AI was carried out by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Hilden, Germany). The prepared enzyme solution was loaded into the column, and treated with a washing buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, 6 M urea, and 2 mM β -mercaptoethanol]. Refolding of the bound protein was conducted by applying a stepwise descending gradient with 6, 4, 2, and 0 M urea including 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, and 2 mM β -mercaptoethanol. The refolded enzyme was eluted initially with a refolding buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, 20% glycerol, and 2 mM β -mercaptoethanol], and ended with elution buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 500 mM imidazole, and 2 mM β -mercaptoethanol]. The molecular mass of L-AI was estimated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as introduced by Laemmli [25].

Enzyme activity assay

The L-AI activity was measured according to the D-tagatose forming property using 10 mM of D-galactose, 25 mM of Tris-HCl buffer (pH 7.5), 1 mM of MnCl₂, and 0.1 mg of the purified enzyme. The enzyme reaction was carried out at 35°C for 30 min, and was stopped by boiling for 10 min. After boiling, the mixture was cooled and centrifuged at 13,000 rpm for 5 min to remove the precipitant. The amount of D-tagatose produced was determined using high-performance liquid chromatography (HPLC) with a refractive index detector (RID) connected to a Sugar-Pak I analytical column (6.5 \times 300 mm; Waters Corp., Milford, MA, USA). The column was eluted by de-ionized water at 78°C at a flow rate of 0.5 mL/min. One unit of L-AI activity was defined as the amount of enzyme producing 1 μ mol of D-tagatose per minute at 35°C and pH 7.5. The concentration of protein was calculated following Bradford's method using bovine serum albumin as the standard [26].

Effect of metal ions on enzyme activity

The effects of metallic ions on L-AI activity were determined by testing various metal ions of MnCl_2 , CoCl_2 , ZnCl_2 , MgCl_2 , CuCl_2 , and CaCl_2 . The purified enzyme was treated with 10 mM of ethylenediaminetetraacetic acid at 4°C for 24 h to make the enzyme metal-free. The enzyme reactions were then carried out in the absence and presence of 1 mM divalent cations at 35°C for 30 min. The effect of the metal ion was determined using the same enzyme assay described above.

Enzyme kinetics

The kinetic parameters of L-AI were determined using D-galactose as a substrate at eight different concentrations (30–600 mM) with the purified enzyme (0.1 mg) in 25 mM Tris-HCl buffer (pH 7.5) and 1 mM of Mg^{2+} . The enzyme reactions were conducted at 50°C for 15 min. The amount of keto-sugar formed was determined by HPLC. Based on these reactions, the initial rates of the bioconversion of D-galactose were identified. The kinetic parameters (K_m and k_{cat}) were investigated via nonlinear regression analysis with SigmaPlot 13.0 software (Systat Software Inc.; San Jose, CA, USA). All assays were carried out in duplicate.

Enzymatic conversion of D-galactose to D-tagatose

The production of D-tagatose using 10 mM of D-galactose as a substrate by ChAI was conducted in 25 mM Tris-HCl buffer (pH 7.5) with 1 mM Mg^{2+} and 1 mg/mL of purified ChAI at 50–70°C for 10 h. Samples were drawn at the defined time and the amount of produced D-tagatose was measured by HPLC.

Statistical analysis

All data are expressed as the mean \pm standard deviation. Significant differences among the treatments were determined by one-way analysis of variance in IBM SPSS Statistics for Windows (version 21.0, IBM Corp., Armonk, NY). Statistical significance was indicated at a confidence level of 95%.

Results

Cloning and expression of the ChAI gene

Based on the fully sequenced genomic DNA of *C. hylemonae* (NCBI accession number: NZ_GG657759.1), the *araA* gene encoding putative L-AI was successfully isolated and amplified by PCR. The amplified DNA fragment of about 1.5 kb was confirmed and compared with the original *araA* sequence in the NCBI database. The L-AI-encoding sequence was successfully constructed in the recombinant expression vector pET28a-ChAI (Fig 1A). The recombinant L-AI expressed in *E. coli* formed an insoluble inclusion body, which was solubilized in guanidine HCl (6 M) resulting in ~72% dissolution of the aggregated recombinant L-AI (Table 1). The solubilized recombinant L-AI was refolded and purified through the Ni-NTA affinity chromatography column. Guanidine HCl-treated insoluble fraction of ChAI showed two distinct protein bands on the SDS-PAGE gel, in which the molecular weights were estimated to be 57 kDa for fully denatured soluble ChAI as shown in the lane 2 and 37 kDa for partially denatured enzyme (Fig 1B). Depending the denaturation conditions, it has been known that degree of protein denaturation might be different [27]. Incomplete denaturation by SDS treatment has also been reported for some thermostable enzymes [28]. The purified L-AI exhibited a specific activity of 0.44 U/mg at 35°C with D-galactose as a substrate.

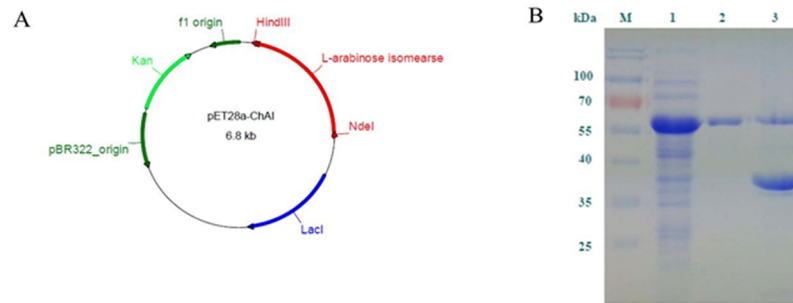


Fig 1. Construction of pET28a-*ChAI* and SDS-PAGE analysis of *ChAI* (B). (A) Map of the plasmid used for the expression of recombinant *ChAI* in *E. coli* BL21 (DE3). (B) Lane M, protein size marker; lane 1, cell extract of *E. coli* BL21 (DE3) harboring 6× His-tagged *ChAI*; lane 2, refolded 6× His-tagged *ChAI* after purification using the Ni-NTA affinity column; lane 3, residual precipitate after solubilization of inclusion bodies in the buffer containing 6 M guanidine hydrochloride.

<https://doi.org/10.1371/journal.pone.0196099.g001>

Effect of temperature on enzyme activity and stability

The temperature profile for *ChAI* activity is shown in Fig 2. The optimal temperature of *ChAI* was 50°C when D-galactose was used as a substrate (Fig 2A). The residual activity of *ChAI* was approximately 81% and 38% at 65°C and at 90°C, respectively. The thermostability of *ChAI* was investigated by measuring the enzyme activity over a temperature range from 40°C to 70°C for 150 min (Fig 2B). *ChAI* displayed remarkable stability with 90% and 85% of the initial activity preserved at 45°C and 60°C, respectively. However, there was a considerable decrease in its activity along with the incubation time at over 65°C. The enzyme deactivation constant (k_d) value for the thermal inactivation of *ChAI* was calculated to be 0.486 s⁻¹ and the half-life ($t_{1/2}$) was 85.6 min at 70°C (Fig 2C).

Effect of pH on enzyme activity and stability

The stable pH profile for the *ChAI* activity of D-tagatose production exhibited quite a wide range, although the optimal pH was determined to be 7.5. Interestingly, *ChAI* could maintain its high-level activity within the acidic pH range (3.0–5.5) as well as within the alkaline pH range (8.0–9.0), with over 80% of its maximum activity retained (Fig 3A). In addition, the pH stability profile for recombinant *ChAI* showed that the enzyme was most stable at pH 6.5–7. It was also stable at a slightly acidic pH, with about 92% relative activity at pH 6.0 (Fig 3B).

Effect of metal ions on enzyme activity

The *ChAI* activity was measured with different types of divalent metal ions (1 mM), and no significant effect on tagatose production was detected by each of Ni⁺, Zn²⁺, Ca²⁺, and Cu²⁺ ions (Table 2). Several studies have reported that Cu²⁺ inhibits L-AI activity; [11, 18, 24, 29] however, this ion significantly enhanced the activity of *ChAI*. In addition, the enzyme activity

Table 1. Purification of 6× His-tagged recombinant *C. hylemonae* L-arabinose isomerase.

Fractions	Total protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)
Total protein	232.59	-	-	-
Inclusion bodies	45.71	-	-	100
Solubilized protein	32.76	-	-	71.7
Ni-NTA	19.33	0.44	8.44	42.3

<https://doi.org/10.1371/journal.pone.0196099.t001>

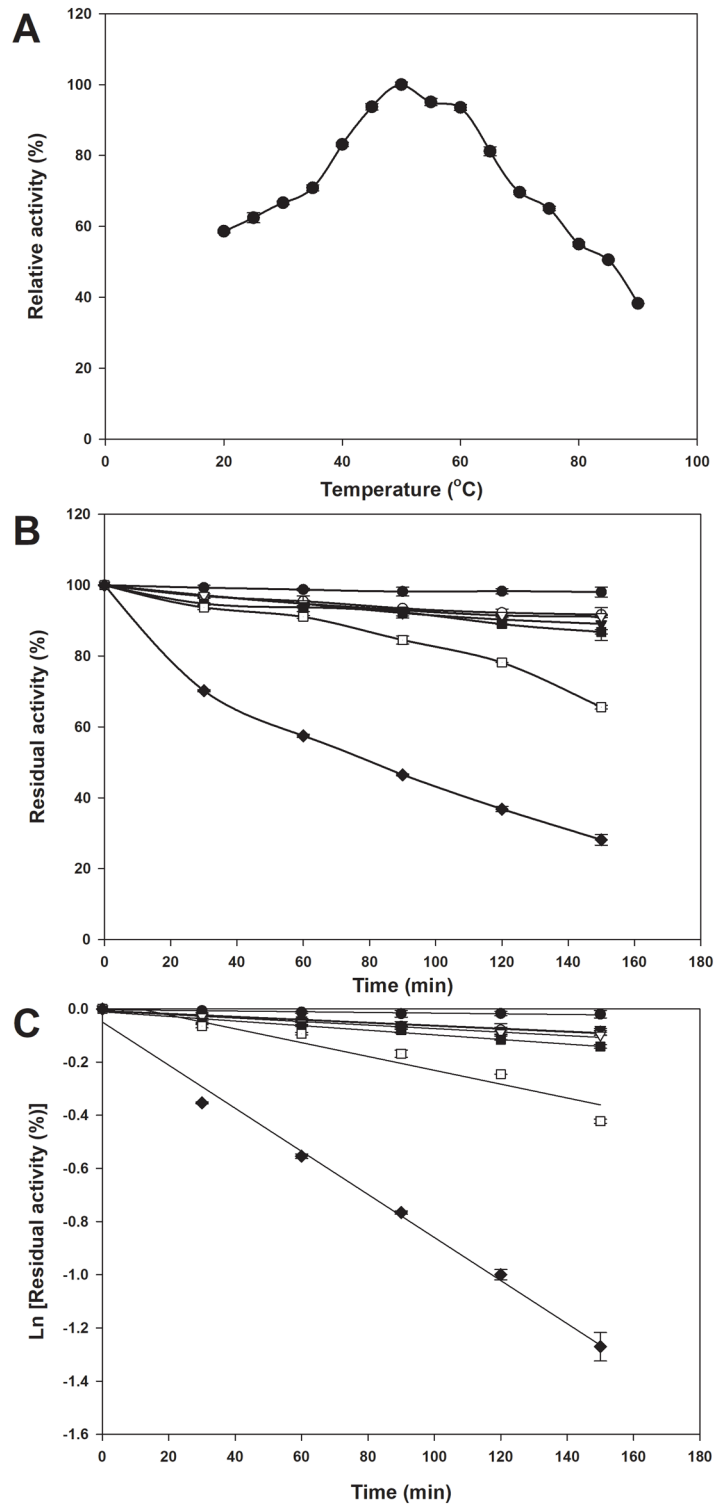


Fig 2. Effect of temperature on the activity and thermal stability of ChAI. (A) The relative enzyme activity was assayed by D-tagatose formation for 30 min. Activity at the optimal temperature was defined as 100%. (B) Thermal stability profile of ChAI at different temperatures: solid circles, 40°C; open circles, 45°C; solid triangles, 50°C; open triangles, 55°C; solid squares, 60°C; open squares, 65°C; solid diamonds, 70°C. The relative enzyme activity was assayed by tagatose production for 150 min. (C) The residual activities were measured at 40–70°C to determine the constant of enzymatic deactivation (k_d). The initial activity was defined as 100%. The values are the means of two independent assays.

<https://doi.org/10.1371/journal.pone.0196099.g002>

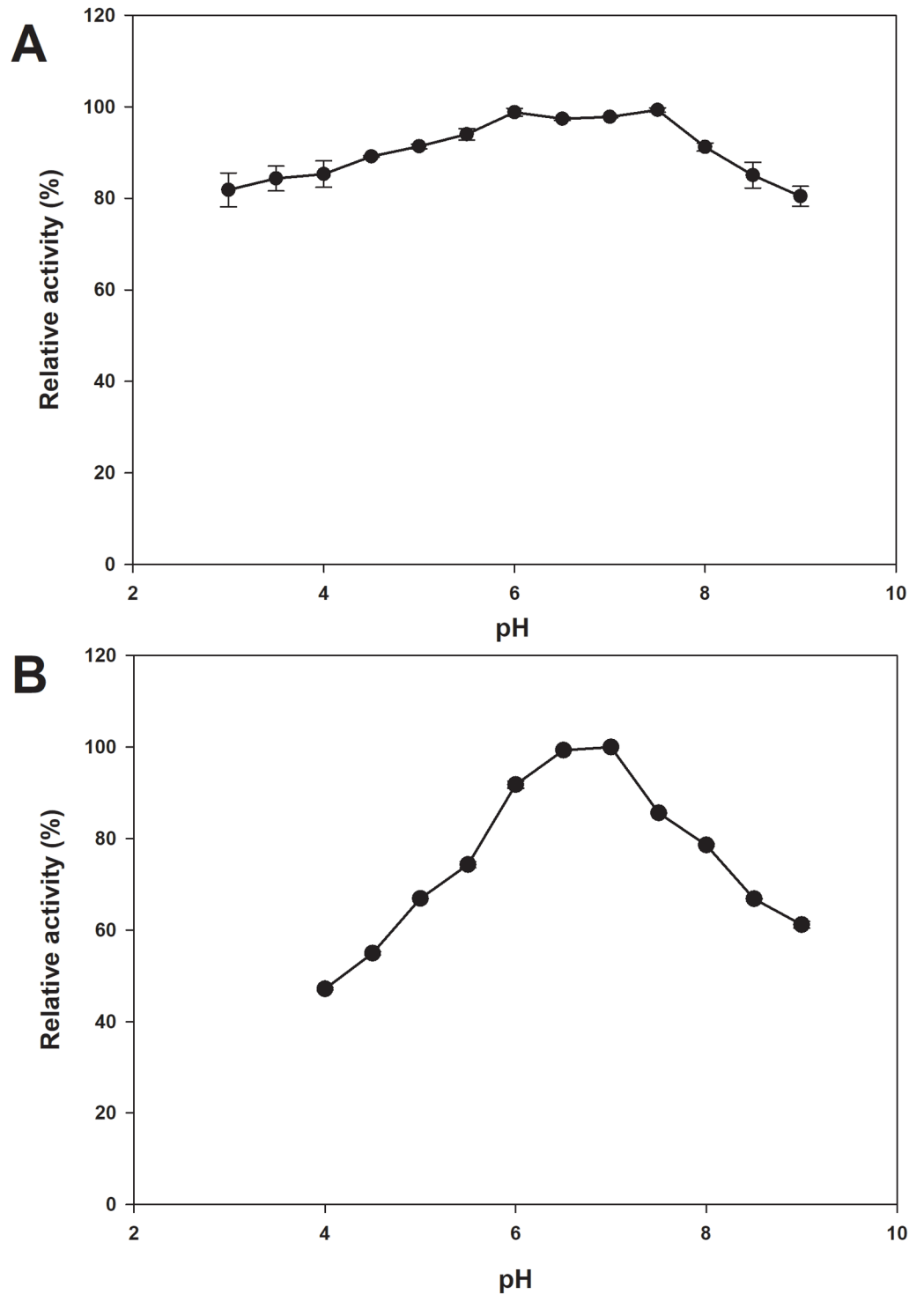


Fig 3. Effect of pH on the enzymatic activity and stability of ChAI. (A) The enzyme activity was determined by monitoring the formation of tagatose from galactose at 35°C for 30 min incubation. (B) The enzyme stability of ChAI was carried out at 4°C for 24 h. The remaining activity was determined in standard assay conditions.

<https://doi.org/10.1371/journal.pone.0196099.g003>

Table 2. Effect of various metal ions on ChAI activity.

Metal ion (1 mM)	Relative activity ¹ (%)
Control ²	100 ^b ± 2
EDTA	0 ^a
Mn ²⁺	111 ^{b,c} ± 2
Co ²⁺	110 ^{b,c} ± 3
Mg ²⁺	120 ^c ± 3
Zn ²⁺	106 ^b ± 3
Ni ²⁺	106 ^b ± 2
Ca ²⁺	103 ^b ± 2
Cu ²⁺	102 ^b ± 3

¹ Values are the means ± standard deviations (n = 2).

Different superscript letters (a-c) associated with values in the same column indicate statistically significant differences (P < 0.05).

² Native enzyme without EDTA treatment and extra metal ion.

<https://doi.org/10.1371/journal.pone.0196099.t002>

was increased by 20% of the relative activity compared to its initial activity in the presence of Mg²⁺. Moreover, 1 mM of Mg²⁺ was found to be the optimal concentration to most effectively enhance the activity of ChAI within the concentration range tested of 0.2 to 2.0 mM (S1 Fig).

Kinetic parameters

ChAI followed a Michaelis-Menten (MM) equation for D-tagatose production from D-galactose (S2 Fig). The apparent MM constant (K_m) was 7.70 mM and the apparent catalytic constant (k_{cat}) was 28.39 sec⁻¹. The kinetics analysis showed that ChAI exhibits higher substrate affinity toward D-galactose compared to L-AIs originating from other microorganisms (Table 3).

Conversion of D-galactose to D-tagatose using ChAI

The bioconversion of D-galactose into D-tagatose using ChAI was conducted at 50–70°C. The amount of D-tagatose reached equilibrium after 2-h incubation (S3 Fig). Interestingly, the production yields of D-tagatose increased from 36.1% to 45.9% as the reaction temperature increased from 50°C to 60°C, and then decreased to 34.4% with a further temperature increase to 70°C after 10 h of the reaction (Fig 4).

Table 3. Kinetic parameters of ChAI reaction.

Microorganism	K _m	k _{cat}	k _{cat} /K _m	Reference
	(mM)	(s ⁻¹)	(s ⁻¹ mM ⁻¹)	
<i>Clostridium hylemonae</i> 15053	7.70	28.39	3.69	This study
<i>Bacillus subtilis</i>	279	53.08	0.19	[36]
<i>Anoxybacillus flavithermus</i>	25.19	2.17	0.09	[37]
<i>B. stearothermophilus</i> US100	8.9	1.26	0.14	[22]
<i>G. stearothermophilus</i> T6	9.0	0.65	0.07	[28]
<i>Thermotoga maritima</i>	18.9	2.68	0.05	[20]
<i>Thermoanaerobacterium saccharolyticum</i> NTOU1	122	4.90	0.04	[19]
<i>Geobacillus thermodenitrificans</i>	408	3.40	0.008	[11]
<i>Thermotoga neapolitana</i>	250	13.50	0.054	[32]

<https://doi.org/10.1371/journal.pone.0196099.t003>

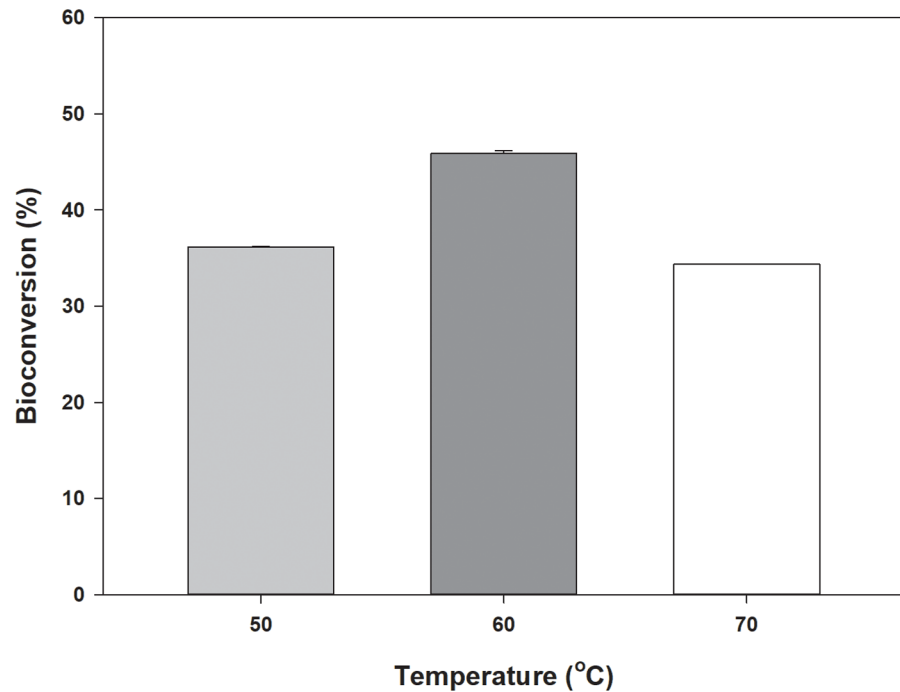


Fig 4. Temperature effect of the *ChAI* reaction on the production yield of D-tagatose. Conversion yields of D-galactose to D-tagatose were carried out at a reaction temperature range between 50°C and 70°C for 10 h. The values are the means of two independent assays.

<https://doi.org/10.1371/journal.pone.0196099.g004>

Discussion

A novel L-AI from *C. hylemonae* was cloned, and its biochemical characteristics were determined focusing on high-value D-tagatose production by isomerization. The newly identified enzyme showed optimal activity at 50°C, pH 7.5, and required Mg^{2+} for enhancing its D-tagatose production property. Moreover, the enzyme displayed remarkable thermostability at 60°C and pH 6. D-Tagatose production by *ChAI* resulted in a high conversion yield of approximately 46% at 60°C. For bio-industrial applications, D-tagatose production must be carried out at temperatures over 60°C, which can increase the bioconversion yield, reduce the viscosity of the reaction mixture, and decrease the possibility of microbial contamination [19, 20, 22, 30]. However, enzyme reactions above 70°C may result in undesirable reactions such as the formation of unexpected byproducts and a browning reaction [22]. Although *ChAI* showed the greatest activity at 50°C, considerable levels of relative activity (93% at 60 min) and thermostability (85% after 150 min) remained at 60°C. Interestingly, the MM constant (K_m) of *ChAI* was lower and the turnover number (k_{cat}) was higher than any other L-AIs reported previously (Table 3). The greatest catalytic efficacy (k_{cat}/K_m : $3.69\text{ s}^{-1}\text{mM}^{-1}$) of *ChAI* for tagatose production suggests that *ChAI* would be a prospective candidate for industrial application at a competitive temperature and production rate.

Although the optimal pH of *ChAI* activity for D-tagatose production was 7.0, it was still remarkably stable over a wide range of pH 3.0–10.0. The acid tolerance of L-AI is an important factor with respect to isomerization under acidic conditions to prevent the formation of inevitable byproducts and other environmental concerns [31]. L-AI from *C. hylemonae* maintained high activity and was sufficiently stable at pH 6.0 (~92% activity), which satisfy the industrial requirements of D-tagatose production as a green technology.

The D-tagatose production by ChAI at 50°C was ca. 35%, which is almost identical to the previous result for a mesophilic L-AI. The reaction toward D-galactose is more dominant in an equilibrium reaction between D-galactose and D-tagatose from a mesophilic L-AI, resulting in a low conversion ratio of D-tagatose [11]. It was previously reported that the bioconversion equilibrium between D-galactose and D-tagatose shifted preferentially toward D-tagatose at higher temperature [20]. In particular, the conversion yield of D-galactose to D-tagatose using the L-AI from *E. coli* was less than 30% at 37°C [32, 33]. However, in the present study, the production yield of D-tagatose reached approximately 46% at 60°C. This specific enzyme displays the greatest conversion catalytic property as a mesophilic L-AI for D-tagatose production reported to date. *Thermotoga neapolitana* L-AI produced D-tagatose from 10 mM of D-galactose with yields of 68% at 80°C, while only 22% of D-tagatose was obtained at 50°C after 20 h [34]. It should be pointed out that within a relatively short reaction time of 6 h, this *Thermotoga* strain showed a bioconversion yield of 56% by the isomerization of D-galactose to D-tagatose at a very high temperature of 80°C [20]. Thus, hyperthermophilic L-AI produced D-tagatose from D-galactose effectively due to an equilibrium shift toward D-tagatose at high reaction temperature [11]. However, the browning reaction above 70°C will negatively affect the final products. Therefore, for commercial production, it may be preferable to utilize L-AI s that act well at around 60°C to limit undesirable color formation. It was previously reported that thermophilic L-AIs exhibited higher conversion yields than hyperthermophilic L-AI s at this temperature as well [34, 35]. Thus, considering the inherent biochemical properties, *C. hylemonae* L-AI could be applied as a potential enzyme for D-tagatose production by improving the protein expression systems (e.g., expression vectors or host) for commercial application.

Supporting information

S1 Fig. Effect of Mg²⁺ concentration on the activity of L-AI from *C. hylemonae*.

(TIF)

S2 Fig. Lineweaver-Burk plot of ChAI using D-galactose as substrate. The assays were conducted with the specified range of D-galactose concentration.

(TIF)

S3 Fig. Effect of the reaction time of ChAI on the production of D-tagatose at various temperatures: (□) 50°C; (○) 60°C; (△) 70°C. The values are the means of two independent assays.

(TIF)

Acknowledgments

This research was supported by High Value-added Food Technology Development Program (Project number: 115035-03-2-HD020), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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Supervision: Moon-Gi Hong.

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Writing – review & editing: Moon-Gi Hong, Byung-Hoo Lee.

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