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

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## 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 $Mg^{2+}$ as a cofactor. Notably, the catalytic efficiency (3.69 $mM^{-1}sec^{-1}$) 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

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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′-GAGACA*GGATCC*ATGATAAAAAGCAAAGAA-3′ (Forward) and 5′-ACATTC*CTCGAG*CT AAATCCCCAGCTTATAGGC-′ (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* (*Ch*AI), was then subcloned into the expression vector pET-28a(+) with the *Nde*I and *Hind*III restriction sites. The sub-cloned pET28a-*Ch*AI 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 ×*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 (Sonics & 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 × *g* at 4˚C for 20 min, and the purification of 6× 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 treateded 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 $MnCl_2$, 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 × 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 μmol 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 *Ch*AI was conducted in 25 mM Tris-HCl buffer (pH 7.5) with 1 mM $Mg^{2+}$ and 1 mg/mL of purified *Ch*AI 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 ± 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 *Ch*AI 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-*Ch*AI (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 *Ch*AI 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 *Ch*AI 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.

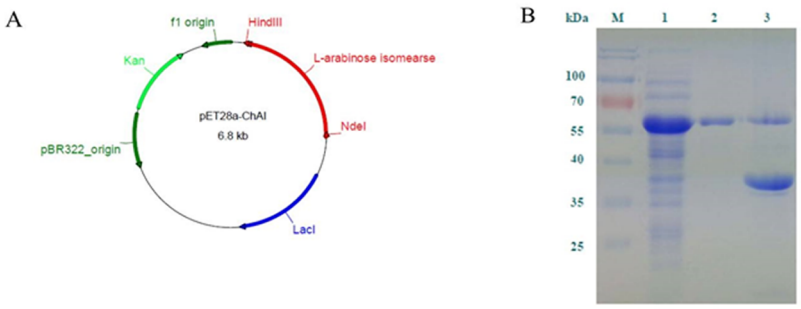


**Fig 1. Construction of pET28a-*Ch*AI and SDS-PAGE analysis of *Ch*AI (B).** (A) Map of the plasmid used for the expression of recombinant *Ch*AI in *E. coli* BL21 (DE3). (B) Lane M, protein size marker; lane 1, cell extract of *E. coli* BL21 (DE3) harboring 6× His-tagged *Ch*AI; lane 2, refolded 6× His-tagged *Ch*AI 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.

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## Effect of temperature on enzyme activity and stability

The temperature profile for *Ch*AI activity is shown in Fig 2. The optimal temperature of *Ch*AI was 50˚C when D-galactose was used as a substrate (Fig 2A). The residual activity of *Ch*AI was approximately 81% and 38% at 65˚C and at 90˚C, respectively. The thermostability of *Ch*AI was investigated by measuring the enzyme activity over a temperature range from 40˚C to 70˚C for 150 min (Fig 2B). *Ch*AI 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 *Ch*AI 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 *Ch*AI activity of D-tagatose production exhibited quite a wide range, although the optimal pH was determined to be 7.5. Interestingly, *Ch*AI 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 *Ch*AI 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 *Ch*AI activity was measured with different types of divalent metal ions (1 mM), and no significant effect on tagatose production was detected by each of $Ni^{+}$, $Zn^{2+}$, $Ca^{2+}$, and $Cu^{2+}$ ions (Table 2). Several studies have reported that $Cu^{2+}$ inhibits L-AI activity; [11, 18, 24, 29] however, this ion significantly enhanced the activity of *Ch*AI. 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 |

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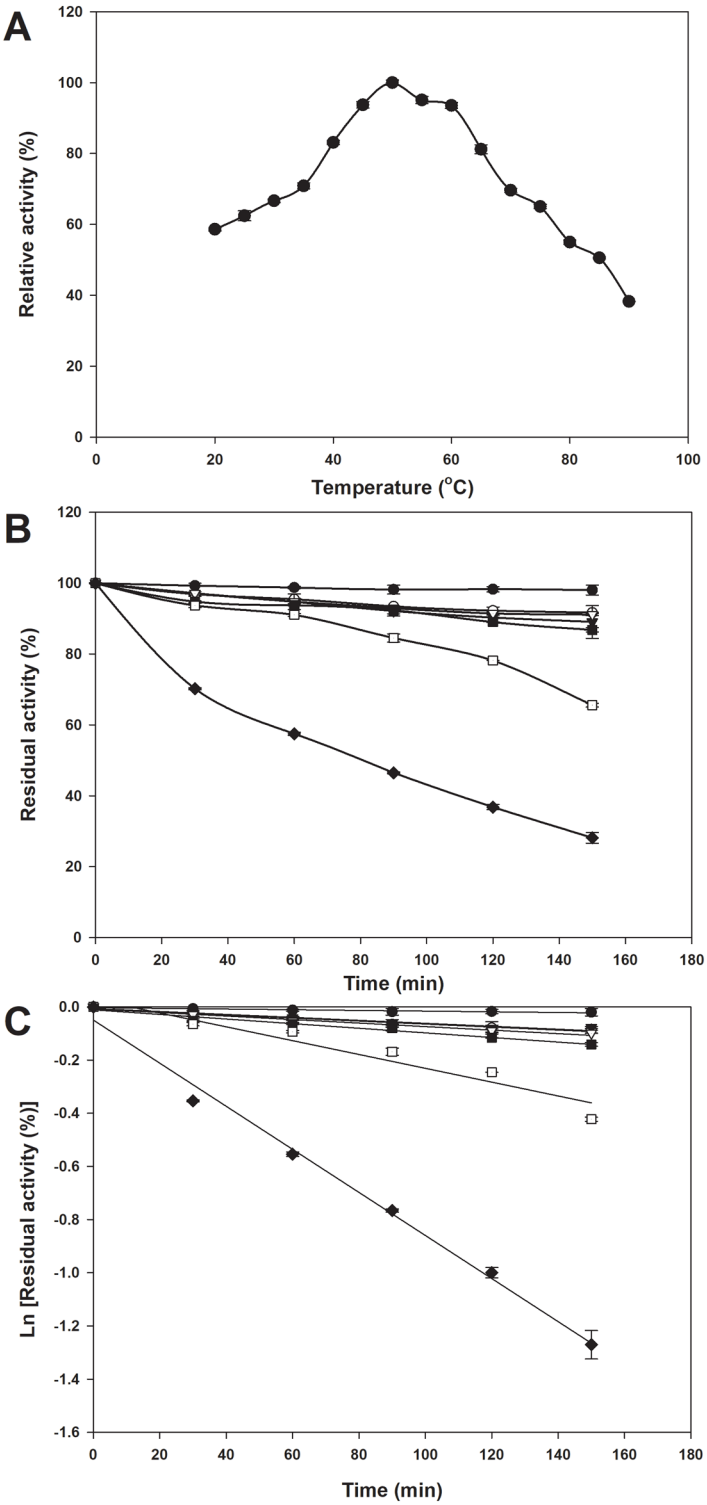


**Fig 2. Effect of temperature on the activity and thermal stability of *Ch*AI.** (A) The relative enzyme activity was assayed by D-tagatose formation for 30 min. Activity at the optimal temperature was defined as 100%. (B) Thermal stability profile of *Ch*AI at different temperatures: solid circles, 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.

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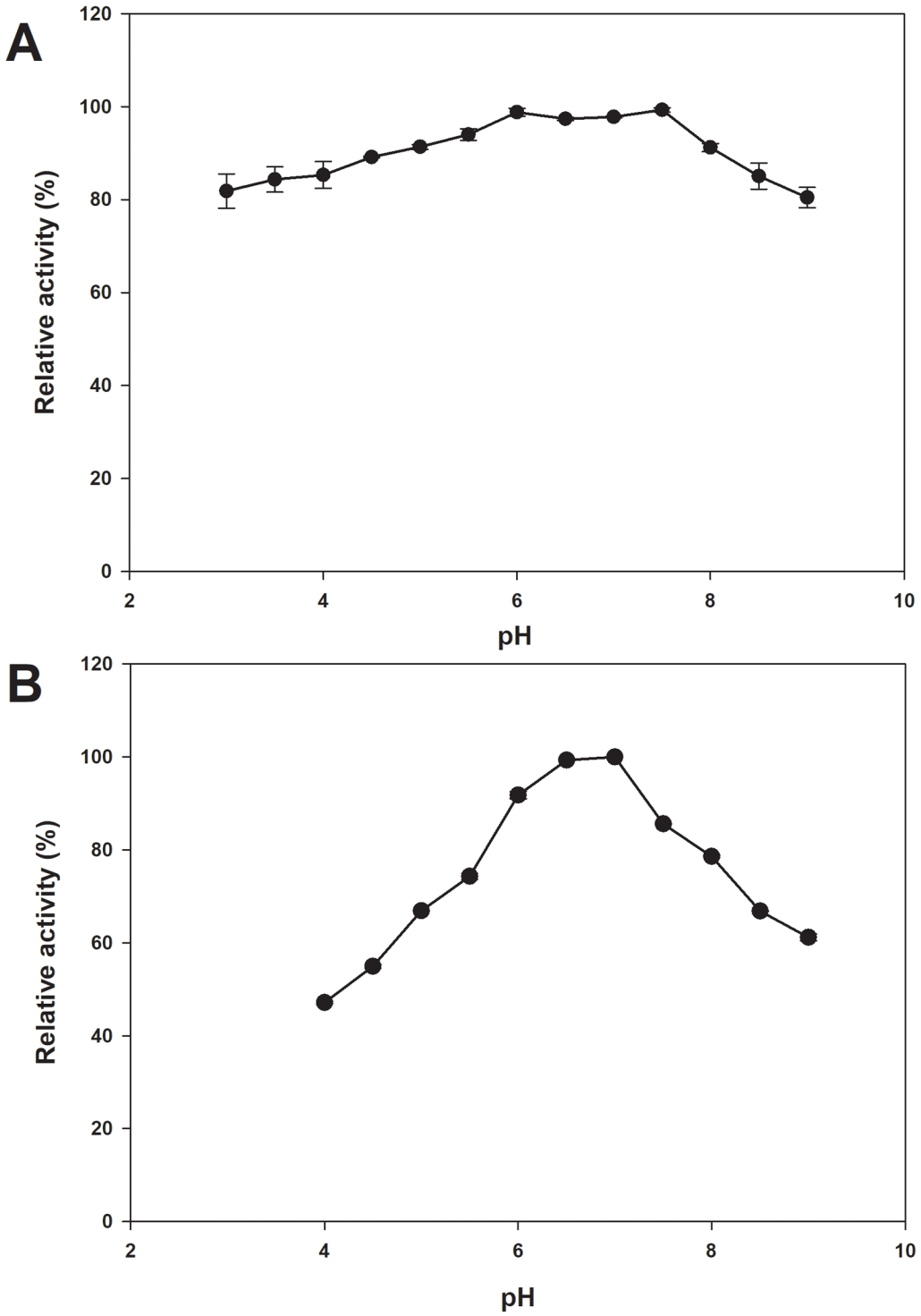


**Fig 3. Effect of pH on the enzymatic activity and stability of *Ch*AI.** (A) The enzyme activity was determined by monitoring the formation of tagatose from galactose at 35˚C for 30 min incubation. (B) The enzyme stability of *Ch*AI was carried out at 4˚C for 24 h. The remaining activity was determined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

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**Table 2. Effect of various metal ions on *Ch*AI activity.**

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

[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.

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was increased by 20% of the relative activity compared to its initial activity in the presence of $Mg^{2+}$. Moreover, 1 mM of $Mg^{2+}$ was found to be the optimal concentration to most effectively enhance the activity of *Ch*AI within the concentration range tested of 0.2 to 2.0 mM (S1 Fig).

## Kinetic parameters

*Ch*AI 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 *Ch*AI 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 *Ch*AI

The bioconversion of D-galactose into D-tagatose using *Ch*AI 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 *Ch*AI reaction.**

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

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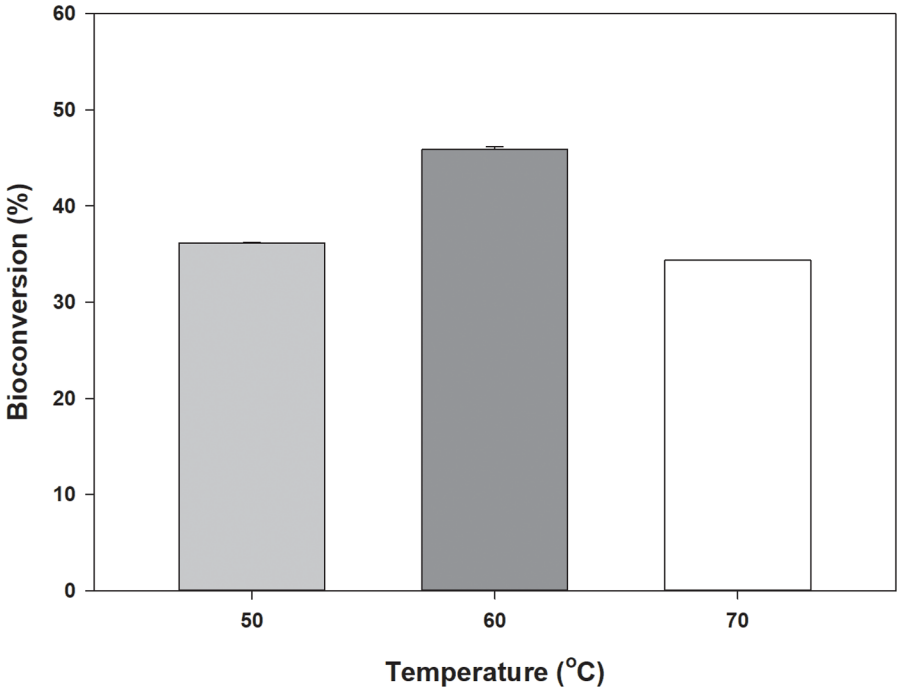


**Fig 4. Temperature effect of the *Ch*AI reaction on the production yield of D-tagatose.** Conversion yields of D-galactose to D-tagatose were carried out at a reaction temperature range between 50˚C and 70˚C for 10 h. The values are the means of two independent assays.

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## 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 *Ch*AI 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 *Ch*AI 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 *Ch*AI 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 $s^{-1}mM^{-1}$) of *Ch*AI for tagatose production suggests that *Ch*AI would be a prospective candidate for industrial application at a competitive temperature and production rate.

Although the optimal pH of *Ch*AI 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 *Ch*AI 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 $Mg^{2+}$ concentration on the activity of L-AI from *C. hylemonae*.**
(TIF)

**S2 Fig. Lineweaver-Burk plot of *Ch*AI 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 *Ch*AI 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)

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## Author Contributions

**Conceptualization:** Pahn-Shick Chang, Byung-Hoo Lee, Sang-Ho Yoo.

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**Formal analysis:** Tien-Kieu Nguyen.

**Funding acquisition:** Sang-Ho Yoo.

**Project administration:** Moon-Gi Hong, Sang-Ho Yoo.

**Supervision:** Moon-Gi Hong.

**Writing – original draft:** Tien-Kieu Nguyen, Moon-Gi Hong, Byung-Hoo Lee, Sang-Ho Yoo.

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