Protein purification, crystallization and preliminary X-ray diffraction analysis of L-arabinose isomerase from *Lactobacillus fermentum* CGMCC2921

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L-Arabinose isomerase (AI) catalyzes the isomerization of L-arabinose to L-ribulose, as well as that of D-galactose to D-tagatose. A thermophilic AI derived from *Lactobacillus fermentum* CGMCC2921 (LFAI) was overexpressed in *Escherichia coli* BL21 (DE3). This enzyme was purified to over 95% purity by nickel affinity, Mono-Q ion-exchange and size-exclusion chromatography. The LFAI protein was crystallized from either 0.1 M bis-tris pH 6.5, 23% PEG 3350, 0.3 M NaCl (form 1 crystals) or 0.1 M bis-tris pH 6.0, 25% PEG monomethyl ether 5000 (form 2 crystals). Diffraction data from form 1 LFAI crystals were collected to 2.80 Å resolution using synchrotron radiation. The form 1 crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 85.11$, $b = 184.57$, $c = 186.26$ Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contained six LFAI subunits, corresponding to a calculated Matthews coefficient of 2.29 Å³ Da⁻¹ and a solvent content of 46.22%.

1. Introduction

L-Arabinose isomerase (AI; EC 5.3.1.4) is able to isomerize the aldose L-arabinose to the ketose L-ribulose. The latter is the precursor of D-xylulose-5-phosphate, an intermediate in the pentose phosphate pathway (Lee *et al.*, 1986). Strikingly, L-ribulose is useful in manufacturing L-ribose, a pharmaceutical raw material that is useful for the production of L-form sugar-based antiviral drugs, including those for the treatment of *Hepatitis B virus* (HBV), *Hepatitis C virus* (HCV), *Human immunodeficiency virus* (HIV) and *Human cytomegalovirus* (HCMV) (Okano, 2009; Hu *et al.*, 2011). Moreover, AI was also found to be active in isomerizing D-galactose to D-tagatose (Cheetham & Wootton, 1993). D-Tagatose is a low-calorie sweetener (1.5 kcal g⁻¹ for humans, 38% that of sucrose) which shows numerous advantages for human health, including lowering blood sugar levels, preventing tooth decay and promoting the growth of intestinal probiotics (Oh, 2007; Buemann *et al.*, 2000; Donner *et al.*, 1999; Levin, 2002; Zehner, 1988; Xu *et al.*, 2014). With the increasing demand worldwide for a healthy diet, developing a suitable sucrose substitute has gained remarkable attention from the food industry. As a unique candidate, D-tagatose received a generally recognized as safe (GRAS) certification from the US Food and Drug Administration in 2003. Recently, AIs from various microorganisms have been cloned, heterologously expressed, purified and characterized (Fig. 1). Species studied include *Escherichia coli*, *Bacillus subtilis*, *Geobacillus stearothermophilus*, *G. thermodenitrificans*, *Lactobacillus sakei* 23K, *L. plantarum* NC8, *Thermotoga maritima*, *T. neapolitana*, *Alicyclobacillus acidocaldarius* and *B. licheniformis* (Yoon *et al.*, 2003; Kim *et al.*, 2002, 2006, 2010; Kim & Oh, 2005; Rhimi *et al.*, 2010; Chouayekh *et al.*, 2007; Lee *et al.*, 2004, 2005; Prabhu *et al.*, 2008). Our team has previously characterized *L. fermentum* AI (LFAI), which is a thermophilic enzyme presenting an optimum reaction temperature of 338 K. This enzyme was also active in slightly acidic conditions. It preserved more than 83% of its original activity at pH 6.0 (Xu *et al.*, 2011). In 2006, Manjasetty and Chance reported the crystal structure of apo-form AI derived from *E. coli* (ECAI). ECAI exhibited a hexameric structure corresponding to a molecular weight of 362 kDa (Manjasetty & Chance, 2006). However, ECAI showed a very high K_m value (1480 mM) for D-galactose, making it less valuable in industrial

applications. Recently, the crystallization and preliminary X-ray crystallographic analysis of *G. kaustophilus* AI have been reported (Cao *et al.*, 2014). However, the currently limited structural information on AI makes it difficult to understand the structural differences between mesophilic and thermophilic AIs and also hinders protein engineering based on rational design. Thus, more crystal structures of AI need to be determined to solve this problem. LFAI shares a sequence identity of 42.2% with ECAI (Fig. 1), making it possible to solve its crystal structure by the molecular-replacement method using the ECAI structure as a search model. In this study, we attempted to express, purify and crystallize the LFAI protein. Furthermore, preliminary X-ray diffraction analysis of LFAI has also been performed.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Cloning and gene expression of LFAI. The genomic DNA of *L. fermentum* CGMCC2921 was isolated using a Gram-positive bacterial genomic DNA-extraction kit (Axygen, USA). The gene encoding LFAI was amplified from genomic DNA by polymerase chain reaction (PCR) using two oligonucleotide primers: forward, 5'-GAGATTCCATATGCGTAAGATGCAAGATTACAAG-3' (the *Nde*I restriction site is underlined), and reverse, 5'-GGAATTC-TTACTTGATGTTGATAAAGTCTGA-3' (a TAA stop codon is included and the *Eco*RI restriction site is underlined). PCR was performed in 1× PCR buffer consisting of 2 mM MgCl₂, 20 ng DNA,

Figure 1

Multiple sequence alignment of AIs from *G. stearothermophilus* (GSAI; GenBank accession No. ABY84698.1), *B. stearothermophilus* US100 (BSAI US100; CAI29261.1), *A. acidocaldarius* (AAAI; AAY68209.1), *T. maritima* (TMAI; NP_228089.1), *E. coli* (ECAI; YP_488368.1), *Shewanella* sp. ANA-3 (SSAI ANA-3; ABK48296.1), *B. licheniformis* (BLAI; KFM93600.1) and *L. fermentum* CGMCC2921 (LFAI; this study; ADJ94948.1). Alignment was carried out using *ClustalX*.

10 pmol primers, 200 μ M dNTP Mix and 2.5 U Ex Taq DNA Polymerase (Takara, Japan) with a total volume of 50 μ l. The PCR product was ligated with the pMD-18T vector and transformed into competent *E. coli* DH5 α cells. The cell clones were cultured and sent for DNA sequencing. The LFAI-encoding gene was then digested from the LFAI-pMD-18T vector by NdeI and EcoRI and ligated with the expression vector pET-28a. In the recombinant pET-28a-LFAI vector, an N-terminal His tag was ligated with the gene encoding LFAI (Table 1). The recombinant vector was transformed into *E. coli* DH5 α competent cells. Single cell clones were picked up and grown in LB medium supplemented with 25 μ g ml⁻¹ kanamycin. The plasmid was extracted from the cells and transformed into *E. coli* BL21 (DE3) competent cells. Single cell clones grown on LB solid medium (supplemented with 25 μ g ml⁻¹ kanamycin) were picked up and cultured at 310 K until the mid-exponential phase was reached (when OD₆₀₀ reached 0.6); isopropyl β -D-1-thiogalactopyranoside

Table 1
Macromolecule-production information.

Source organism	<i>L. fermentum</i> CGMCC2921
Forward primer†	GAGATTCATATCGTAAAGTGAAGTACAAG
Reverse primer‡	GGAATCTTACTTGTATGTGATAAGTCGA
Cloning vector	pMD-18T
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced§	MGSSHHHHSSGLVPRGSHMRKMQDYKFWFVVGSPPLYGPE- ALAEVEKDARKLVDGLNKGKLDYPVEFKLVATTADSITK- FMKEANYNDVAGVITWMHTFSPAKNWIRGTELLQKPLLH- LATQFLNNIPFDSIDMDYMLHQAHDREYAYINSRLNV- PAASVYGGWGDADVQEQIADWQHVAAYNESFHIKARFG- DTMRDVAVTGDKVAAQIKLGWTVDYPTNELVAVVNGIA- EDEIDAAYKDLAENYDLVEGDNDHEKYVHVNRVYQLREYLG- IKKFLDDNGYDAFTDNFQDLEGLQLPLAVQLLMIDGYG- FGPEGDFKMAGLTRLLKIAADNKQALMEDYTLDRHGHE- AIMGSHMLEVDPTLASDKPRVEVHPLGIGGKDDPARLVFT- GAEGKGYDITLSYFDGKYKIGYPVDCKTPEAEMPKLPVA- KQMWTEIGLAEGAKQMKYGGGHHTVLTALSEEQLEQL- ARLFKVDVFINIK

† The NdeI restriction site is underlined. ‡ The EcoRI restriction site is underlined. § His-tag and vector residues are underlined.

Figure 2
(a) Purification of the LFAI protein by Mono Q ion-exchange chromatography. The blue curve shows the UV absorption observed at 280 nm and the red curve shows the conductivity, while the green curve shows the concentration of elution buffer. (b) Purification of the LFAI protein by size-exclusion chromatography. The enzyme activity of the major peak protein was determined by the cysteine-carbazole-sulfuric acid method. The purity of the protein was further measured by SDS-PAGE.

(IPTG) was then added to a final concentration of 1 mM. The cells were grown for an additional 24 h at 293 K and then harvested by centrifugation (5000g, 20 min) and stored at 253 K until use.

2.1.2. Purification of LFAI. The *E. coli* cells (2 l culture medium) expressing LFAI were resuspended in 50 ml lysis buffer [50 mM Tris-HCl pH 7.4, 500 mM NaCl, 5 mM imidazole, 10% (v/v) glycerol] and then disrupted by sonication (400 W, 277 K) for 200 cycles of 3 s followed by cooling for 5 s. The lysate was centrifuged at 20 000g for 50 min (277 K) and the supernatant was collected and loaded onto an Ni-NTA agarose affinity column (5 ml; GE Healthcare, Uppsala, Sweden). The column was then washed with 50 mM Tris-HCl pH 7.4 buffer containing 500 mM NaCl, 50 mM imidazole, 10% (v/v) glycerol until the UV absorption reached zero, and the target protein was then eluted with 200 mM imidazole in lysis buffer. The LFAI protein was loaded onto a Mono Q anionic ion-exchange column pre-equilibrated with 50 mM Tris-HCl pH 7.5, 10% (v/v) glycerol. Protein with AI activity was eluted linearly with elution buffer: 50 mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 1 M NaCl (Fig. 2a). The protein was then desalted using a G25 desalting column (GE Healthcare, Uppsala, Sweden). To further enhance the purity of the AI enzyme, the protein was loaded onto a size-exclusion chromatography column (Superdex S200 16/60, GE Healthcare, Uppsala, Sweden) pre-equilibrated with 10 mM HEPES pH 7.0, 10% (v/v) glycerol, 150 mM NaCl. The peak fractions were collected and analyzed by SDS-PAGE (Fig. 2b). The enzyme activity was also determined for each fraction using the cysteine-sulfuric acid-carbazole method (Dische & Borenfreund, 1951). The active LFAI protein was concentrated to a final concentration of 26.7 mg ml⁻¹ (total amount of protein: 13.2 mg) with an enzyme activity of 9.6 U mg⁻¹ (at 338 K) for D-galactose. The enzyme was flash-cooled using liquid nitrogen and stored at 193 K. To determine the molecular weight of LFAI, standard protein molecular-weight samples (Bio-Rad, Hercules, California, USA; bovine thyroglobulin, 670 kDa; bovine γ -globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B₁₂, 1.35 kDa) were loaded onto the Superdex S200 10/300 size-exclusion chromatography column (GE Healthcare, Uppsala, Sweden). The molecular weight of the LFAI protein was calculated according to a standard curve drawn on the basis of the elution volume and the logarithm of the molecular weight of the protein samples (Fig. 3).

2.1.3. Enzyme assay. AI activity was measured by determination of the amount of L-ribulose (or D-tagatose) formed. The generated L-ribulose (or D-tagatose) was determined by the cysteine-carbazole-

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	10 mM HEPES pH 7.0, 10% (v/v) glycerol, 150 mM NaCl
Composition of reservoir solution	0.1 M bis-tris pH 6.5, 23% PEG 3350, 0.3 M NaCl or 0.1 M bis-tris pH 6.0, 25% PEG monomethyl ether 5000
Volume and ratio of drop	3 µl (1:1 ratio of protein solution and reservoir solution)
Volume of reservoir (µl)	500

sulfuric acid method: 1 ml diluted reaction solution was mixed with 200 µl 1.5% cysteine hydrochloride followed by 6 ml 75% (v/v) concentrated sulfuric acid and then 200 µl of a 0.12% alcoholic solution of carbazole. The absorbance of the mixture was measured at 560 nm (Dische & Borenfreund, 1951). The concentration of L-ribulose (or D-tagatose) generated by the reaction was calibrated based on a standard linear graph prepared using different concentrations of L-ribulose (or D-tagatose). One unit of AI activity was defined as the amount of enzyme catalyzing the formation of 1 µmol keto sugar per minute under the conditions specified above.

2.2. Crystallization

2.2.1. Crystallization of LFAI. To screen for crystals of LFAI, the enzyme was diluted to concentrations of 5, 10, 15 and 20 mg ml⁻¹. Commercial crystallization screening reagents from Hampton Research (Index Screen, Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, SaltRx and SaltRx 2), Molecular Dimensions (Structure Screen I & II), Qiagen (The Protein Complex and PEGs II Suites) and Emerald Bio (Wizard III and IV) were used. The preliminary crystallization screen was performed using the sitting-drop vapour-diffusion method in 96-well plates, with each well containing 75 µl reservoir solution and with drops consisting of 1 µl protein solution mixed with 1 µl reservoir solution. The protein was screened at different concentrations. After 4–5 d at 291 K, microcrystals were found in the following conditions: Index Screen No. 46 [0.1 M bis-tris pH 6.5, 20% (w/v) PEG monomethyl ether 5000], No. 70 [0.2 M sodium chloride, 0.1 M bis-tris pH 5.5, 25% (w/v) PEG 3350], No. 71 [0.2 M sodium chloride, 0.1 M bis-tris pH 6.5, 25% (w/v) PEG 3350] and No. 78 [0.2 M ammonium acetate, 0.1 M bis-tris pH 5.5, 25% (w/v)

Figure 3

The molecular-weight standard curve calculated using a Superdex S200 10/300 size-exclusion chromatography column. The y axis indicates the logarithm of the protein molecular weight of standard protein samples. The x axis indicates the elution volume of each protein.

PEG 3350], SaltRx No. 65 (2.5 M ammonium sulfate, 0.1 M bis-tris propane pH 7.0) and The Protein Complex Suite No. 9 [0.2 M sodium chloride, 0.1 M MES pH 6.0, 20% (w/v) PEG 2000 monomethyl ether], No. 28 [0.2 M lithium sulfate, 0.1 M MES pH 6.0, 20% (w/v) PEG 4000] and No. 91 [15% (v/v) 2-methyl-2,4-pentanediol, 2% (w/v) PEG 4000]. To refine these conditions, the concentration of the crystallization reagent, the pH of the buffer system and the type and

(a)

(b)

Figure 4

Crystals of LFAI in two forms. Hexagonal-shaped form 1 crystals (a) were grown in 0.1 M bis-tris pH 6.5, 23% PEG 3350, 0.3 M NaCl, while form 2 crystals (b) were grown in 0.1 M bis-tris pH 6.0, 25% PEG monomethyl ether 5000. Diffraction data were only obtained from the form 1 crystals, while the form 2 crystals diffracted poorly. (c) Size-exclusion chromatography curves of LFAI purified using Superdex S200 10/300 (peak 1) and of washed crystals (peak 2). An SDS-PAGE gel of the purified LFAI enzyme and washed crystals is also shown.

concentration of the salt were varied and the crystallization process was carried out using the hanging-drop vapour-diffusion method in 24-well plates, with each well containing 500 μl reservoir solution and with drops consisting of 1.5 μl protein solution mixed with 1.5 μl reservoir solution. Finally, we found large single crystals in two optimized conditions (protein concentration of 10 mg ml^{-1}) after one week at 291 K: crystal form 1 was obtained using 0.1 M bis-tris pH 6.5, 23% PEG 3350, 0.3 M NaCl (Fig. 4a) and crystal form 2 was obtained using 0.1 M bis-tris pH 6.0, 25% PEG monomethyl ether 5000 (Fig. 4b). SDS-PAGE analysis and size-exclusion chromatography of washed crystals of both forms 1 and 2 were carried out. As shown in Fig. 4(c), the results indicated that the molecular weights of the whole enzyme and the subunit of the washed LFAI crystal were in accordance with the purified LFAI enzyme. After screening several cryoprotectants, both form 1 and form 2 crystals were found to be stable in a solution consisting of 20% (v/v) glycerol plus 80% reservoir solution. The crystals were picked up in a nylon loop and then soaked for 5–10 s in cryoprotectant solution and flash-cooled in liquid nitrogen before diffraction using synchrotron radiation. The form 2 crystals exhibited poor diffraction, while the form 1 crystals diffracted to 2.8 Å resolution. Thus, data were collected from a form 1 crystal. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

2.3.1. Diffraction experiments. Prior to data collection, the form 1 crystals were picked up in a cryoloop (0.1–0.2 mm; Hampton Research), immediately soaked in cryoprotectant solution for 1–2 s and then flash-cooled in liquid nitrogen. Diffraction data were collected on an ADSC Q315r detector at 100 K on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF). A total of 230 images were collected at a crystal-to-detector distance of 200 mm with 0.5 s exposure for every 1° oscillation frame. Intensity data were integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 3. Model building

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97915
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	0.4
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 85.11, b = 184.57, c = 186.26,$ $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.80
Resolution range (Å)	48.44–2.80
Total No. of reflections	336170
No. of unique reflections	47550
Completeness (%)	98.6 (98.9)
Multiplicity	5.1 (4.7)
$\langle I/\sigma(I) \rangle$	6.2 (2.1)
$R_{\text{r.i.m.}}$ (%)	11.2 (43.6)
Overall B factor from Wilson plot (Å ²)	43.23

and refinement are in progress using *Coot* (Emsley & Cowtan, 2004) and *PHENIX* (Adams *et al.*, 2002).

3. Results and discussion

We successfully cloned, solubly expressed and purified LFAI. The purified active protein showed over 95% purity as judged by SDS-PAGE. To date, only the crystal structure of ECAI is available among the reported AIs (Manjasetty & Chance, 2006). The previously reported AIs can be divided into three types: mesophilic, thermophilic and hyperthermophilic AIs. Most thermophilic and hyperthermophilic AIs have been reported to be tetrameric, including those derived from *T. maritima*, *G. stearothermophilus* US100, *Thermoanaerobacter mathranii* and *G. thermodenitrificans* (Lee *et al.*, 2004; Rhimi & Bejar, 2006; Jørgensen *et al.*, 2004; Kim & Oh, 2005). In contrast, the mesophilic AIs favoured a hexameric tertiary arrangement (Xu *et al.*, 2014). Although LFAI has good stability at high temperatures (Xu *et al.*, 2011), its tertiary structure was shown to be hexameric as determined by size-exclusion chromatography in this study. As observed in the diffraction experiments, the form 2 crystal of LFAI diffracted poorly (~ 5 Å) and was fragile, which might owing to a high solvent content (60.17%) in the crystal. For the form 1 crystal, a total of 47 550 reflections were observed in the resolution range 48.4–2.8 Å (Fig. 5). This crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 85.11$, $b = 184.57$, $c = 186.26$ Å, $\alpha = \beta = \gamma = 90^\circ$. We attempted to use molecular-replacement methods to solve the structure of LFAI using the crystal structure of ECAI as a search model (PDB entry 2ajt; Manjasetty & Chance, 2006). The asymmetric unit contained six LFAI subunits, corresponding to a calculated Matthews coefficient of $2.29 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 46.22% (Matthews, 1968). The monomeric LFAI consists of 474 amino-acid residues with a predicted molecular weight of 53.4 kDa and a pI value of 4.82. Size-exclusion chromatography revealed that the purified His-tagged LFAI in this study possesses a hexameric structure (~ 329 kDa), which correlated well with the predicted molecular weight (343.5 kDa) of the homo-hexameric complex. Moreover, a sixfold peak was observed in the self-rotation function at $\chi = 60^\circ$ (z axis; Supplementary Fig. S1). Further improvement of the LFAI diffraction resolution is currently in progress using crystal dehydration methods and site-directed mutagenesis on the protein surface.

Figure 5
Diffraction image of LFAI obtained on SSRF beamline BL17U.

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