Crystallographic studies of aspartate racemase from *Lactobacillus sakei* NBRC 15893

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Aspartate racemase catalyzes the interconversion between L-aspartate and D-aspartate and belongs to the PLP-independent racemases. The enzyme from the lactic acid bacterium *Lactobacillus sakei* NBRC 15893, isolated from *kimoto*, is considered to be involved in D-aspartate synthesis during the brewing process of Japanese sake at low temperatures. The enzyme was crystallized at 293 K by the sitting-drop vapour-diffusion method using 25%(v/v) PEG MME 550, 5%(v/v) 2-propanol. The crystal belonged to space group $P3_121$, with unit-cell parameters $a = b = 104.68$, $c = 97.29$ Å, and diffracted to 2.6 Å resolution. Structure determination is under way.

1. Introduction

D-Amino acids are known to be essential components of the peptide glycan in the microbial cell wall (Osborn, 1969). However, they were once considered to be unnatural and unusual compounds that were not essential amino acids in mammals, including humans. Recently, D-amino acids have been found in a much broader range of living organisms with the development of improved detection and analytical techniques (Hashimoto & Oka, 1997), and are known to play a variety of important roles. For example, D-aspartate has been found to have an important role in the regulation of developmental processes of the endocrine and/or neuroendocrine organs (Ota *et al.*, 2012). D-Serine has been found to play a significant role in the mammalian central nervous system (Wolosker, 2006). D-Alanine is located in the islets of Langerhans in rat pancreas and might participate in the regulation of mammalian blood-glucose levels (Morikawa *et al.*, 2007). In addition, D-amino acids have been detected in many foods, including vegetables (Brückner & Westhauser, 2003), fruits (Brückner & Westhauser, 2003), milk (Rubio-Barroso *et al.*, 2006), orange juice (Brückner & Lüpke, 1991), beer (Erbe & Brückner, 2000), wine (Ali *et al.*, 2010) and cocoa (Pätzold & Brückner, 2006).

Recently, it has been reported that Japanese sake made by *kimoto*, which is a traditional method of making sake mash, contains high amounts of several D-amino acids (Gogami, Okada & Oikawa, 2011) and that the concentrations of the D-amino acids relate to the taste of sake (Okada *et al.*, 2013). In sake, D-Asp, D-Glu, D-Ala and D-Lys are produced by lactic acid bacteria, and amino-acid racemases of the bacteria are considered to be involved in the synthesis of the D-amino acids (Gogami, Okada, Yano *et al.*, 2011; Gogami *et al.*, 2012). During the brewing process of sake by *kimoto*, the temperature of the sake starts at about 279 K and gradually rises to

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about 296 K over the period of about one month. In the middle period of the process, lactic acid bacteria grow in a temperature range of about 288–293 K and produce lactic acid, preventing the growth of other bacteria (Ohbayashi & Kitahara, 1959). In fact, the lactic acid bacterium *Lactobacillus sakei* NBRC 15893 was isolated from *kimoto* in the brewing process of sake (Katagiri *et al.*, 1934). *L. sakei* has an optimal growth temperature of 303 K and is able to grow and survive at the low temperature of 277 K (Marceau *et al.*, 2003). The aspartate racemase from *L. sakei* NBRC 15893 (LsAspR) has been cloned and overexpressed in *Escherichia coli* (Gogami *et al.*, 2012). The enzyme exists as a homodimer. Its polypeptide chain consists of 234 amino-acid residues and has a mass of 26 240 Da. LsAspR is expected to have different structural features from those of the thermophilic enzyme owing to the adaptation of the enzyme to a low-temperature environment.

Amino-acid racemases are responsible for the racemization of amino acids and can be mainly categorized into two groups: the pyridoxal 5'-phosphate (PLP)-dependent and PLP-independent groups (Conti *et al.*, 2011). Aspartate racemase catalyzes the interconversion between L-aspartate and D-aspartate and belongs to the PLP-independent racemase group (Yamauchi *et al.*, 1992). The crystal structure of the enzyme from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 (PhAspR) is the only example of an aspartate racemase structure reported to date (Liu *et al.*, 2002). Aspartate racemase is thought to employ a two-base mechanism to catalyze both directions of racemization and to utilize two cysteine residues as the conjugated catalytic acid and base in the enzymatic reaction (Yamauchi *et al.*, 1992; Liu *et al.*, 2002). In the reaction mechanism, the roles of two cysteine residues as the catalytic acid and base are switched depending on the enantiomer of the substrate, L-aspartate or D-aspartate. From the crystal structure of PhAspR complexed with citric acid, the possibility of another reaction mechanism, a 'one-base' mechanism, was proposed by regarding the binding citric acid as a dual-substrate analogue (Ohtaki *et al.*, 2008). In the proposed mechanism, each of the two cysteine residues plays an individual catalytic role for both enantiomers of the substrate. The reaction mechanism of aspartate racemase still remains unclear.

Here, we report the crystallization and preliminary X-ray diffraction studies of the aspartate racemase from *L. sakei* NBRC 15893 (LsAspR). The X-ray crystal structure of the present enzyme is expected to elucidate the structure–function relationship of aspartate racemase, which works in the low to medium temperature range. Moreover, the structure of this amino-acid racemase related to fermented foods will provide useful information for extensive studies on D-amino acids in the fields of food science and food engineering.

2. Materials and methods

2.1. Macromolecule production

L. sakei NBRC 15893 acquired from the Biological Resource Center, NITE (NBRC) was cultivated at 303 K and

the genomic DNA was extracted from the bacterium. In order to find the open reading frame for an aspartate racemase homologue gene from *L. sakei* NBRC 15893, the forward primer 5'-CGCACGTTATTTTAAAGAAAATAAAG-3' and the reverse primer 5'-GCCGTGTCTAACAATTACTAAATTC-3' were designed based on the genome sequence of *L. sakei* strain 23k. The amplified DNA fragments contained an aspartate racemase homologue gene with redundant regions at both ends. The first PCR amplification was carried out on the *L. sakei* NBRC 15893 genome. The PCR products were cloned into the pT7Blue-2 T vector (Novagen). The cloned DNA sequence was confirmed by nucleotide-sequence analysis. To construct the overproduction system of the enzyme, the forward primer 5'-**CATATGAAACAGTTTTT**-TACAGTGCTCG-3' containing an NdeI site (shown in bold) and the reverse primer 5'-**CTCGAGCTTTT**CACGTAACCTTAAGGC-3' containing an XhoI site (shown in bold) were designed from the confirmed open reading frame sequence of the LsAspR gene. The second PCR was performed on the *L. sakei* NBRC 15893 genome. The amplified PCR products were subcloned with the pT7Blue-2 T vector and the sequences of the amplified DNA fragments were confirmed by nucleotide-sequence analysis. The fragments were ligated into an NdeI- and XhoI-digested pET-21b vector (Novagen) and the constructed plasmid was transformed into *Escherichia coli* BL21 (DE3) cells.

E. coli BL21 (DE3) cells harbouring the recombinant plasmid pET-21b carrying the LsAspR gene were grown in ampicillin-containing LB medium at 310 K. When the optical density reached approximately 0.6 at 600 nm, IPTG was added to the culture medium to a final concentration of 0.5 mM. The cultures were placed at 288 K and cultivated for 24 h. After cultivation, the cells were collected by centrifugation (14 000g, 10 min, 277 K) and washed twice with 0.75% (w/v) NaCl solution. The washed cells were suspended in buffer A [10 mM imidazole, 300 mM NaCl, 0.01% (v/v) β -mercaptoethanol, 50 mM sodium phosphate buffer pH 8.0] and disrupted with an ultrasonic disintegrator. The cell debris was removed by centrifugation (26 100g, 20 min, 277 K) to obtain the crude enzyme in the supernatant solution.

Purification of the C-terminally His-tagged protein was performed by chromatography at 277 K. The crude enzyme was applied onto an Ni-NTA Superflow (Qiagen) column equilibrated with buffer A and washed with buffer B [60 mM imidazole, 300 mM NaCl, 0.01% (v/v) β -mercaptoethanol, 50 mM sodium phosphate buffer pH 8.0]. The adsorbed proteins were eluted with buffer C [250 mM imidazole, 300 mM NaCl, 0.01% (v/v) β -mercaptoethanol, 50 mM sodium phosphate buffer pH 8.0]. The eluted protein fractions showing a peak at 280 nm absorbance were combined and concentrated by ultrafiltration. The concentrated protein solution was applied onto a PD-10 desalting column (GE Healthcare) equilibrated with 50 mM sodium phosphate buffer pH 6.5, 4 mM dithiothreitol. The LsAspR protein was eluted with the same buffer. The purity of the eluted protein was determined by 12% (w/v) SDS-PAGE analysis. Macromolecule-production information is summarized in Table 1.

Table 1

Macromolecule-production information.

Source organism	<i>L. sakei</i> NBRC 15893
DNA source	Genomic DNA
Forward primer†	CATATGAACAGTTTTCACAGTGCTCG
Reverse primer‡	CTCGAGCTTTTCACGTAACCTAAGGC
Cloning vector	pT7Blue-2 T
Expression vector	pET-21b
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced§	MKQFFTVLGGMGTAATESYIRLLNQRTPTTKDQD- FLNYIVVNHATIPDRSTYLMDSQSPSPDLL- EDIQQSLLKPAFFVIACNTAHFYDDLQAAA- SAPIVHMPRETVKSITQTYPAKRIGILGTGK- TVTDGIYDEALLAEGYEVVKPSLDLQERTMDL- IFNDIKGQSQMKPEKYHAILAEMQTQCDAIL- GCTELSLAQEWAPDHPVVDQSQSVLVDRSIE- LGLKLEKLEHHHHHH

† The underlined sequence corresponds to the NdeI site. ‡ The underlined sequence corresponds to the XhoI site. § The underlined sequence corresponds to the His-tag site.

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion with micro-seeding
Plate type	MRC2 96-well crystallization plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	20
Buffer composition of protein solution	50 mM MES buffer pH 6.5, 4 mM dithiothreitol
Composition of reservoir solution	25%(v/v) PEG MME 550, 5%(v/v) 2-propanol, 0.1 M sodium acetate pH 4.8
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	100

2.2. Crystallization

Initial crystallization experiments were performed by the sitting-drop vapour-diffusion method using Crystal Screen, Crystal Screen 2, PEGRx 1 and PEGRx 2 (Hampton Research) at 293 K. The protein concentration was adjusted to 20 mg ml⁻¹ in 50 mM MES buffer pH 6.5, 4 mM dithiothreitol. Each of the drops consisting of 1 µl protein solution and 1 µl reservoir solution was equilibrated against 100 µl reservoir solution. Small crystals were obtained in several days using PEGRx 2 solution No. 10. The crystallization conditions were optimized based on this solution. Consequently, a 2.0 µl protein drop containing micro-seed crystals was equilibrated

Figure 1

A bundle of crystals of aspartate racemase from *L. sakei* NBRC 15893.

against 100 µl reservoir solution consisting of 25%(v/v) polyethylene glycol monomethyl ether (PEG MME) 550, 5%(v/v) 2-propanol, 0.1 M sodium acetate pH 4.8. The droplet was prepared by mixing 1 µl 20 mg ml⁻¹ protein solution (in 50 mM MES buffer pH 6.5, 4 mM dithiothreitol) and 1 µl reservoir solution. Bundles of rod-shaped crystals were finally produced at 293 K in 3 d by using the sitting-drop vapour-diffusion method supplemented with a micro-seeding technique (Fig. 1). The crystallization information is summarized in Table 2.

2.3. Data collection and processing

Diffraction experiments were performed on beamline NE-3A at Photon Factory PF-AR, Tsukuba, Japan. A bundle of rod-shaped crystals was divided into single rod-shaped crystals in a droplet of the reservoir solution with a nylon loop used for crystal mounting. A single rod-shaped crystal with dimensions of 0.35 × 0.06 × 0.03 mm was flash-cooled in a nitrogen stream at 100 K. No cryoprotectant was used in the cooling because the concentration of the precipitant reagents in the reservoir solution was sufficiently high to prevent the formation of ice. Diffraction data were collected at a wavelength of 1.000 Å using an ADSC Quantum 270 CCD detector at a crystal-to-detector distance of 331.2 mm. A data set was collected using 180 frames, each of which covered an 1.0° oscillation range and had an exposure time of 2.0 s (Fig. 2). All diffraction images were processed to 2.6 Å resolution with *iMosflm* (Battye *et al.*, 2011) and *SCALA* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011). The statistics of data collection and processing are summarized in Table 3.

Figure 2

X-ray diffraction image from a crystal of aspartate racemase from *L. sakei* NBRC 15893. The maximum resolution processed was 2.6 Å.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	NE-3A, PF-AR
Wavelength (Å)	1.000
Temperature (K)	100
Detector	ADSC Quantum 270 CCD
Crystal-to-detector distance (mm)	331.2
Rotation range per image (°)	1.0
Total rotation range (°)	180
Exposure time per image (s)	2.0
Space group	$P3_121$
Unit-cell parameters (Å, °)	$a = b = 104.68$, $c = 97.29$, $\alpha = \beta = 90$, $\gamma = 120$
Mosaicity (°)	1.07
Resolution range (Å)	52.34–2.55 (2.69–2.55)
Total No. of reflections	209960 (26315)
No. of unique reflections	20450 (2934)
Completeness (%)	100.0 (100.0)
Multiplicity	10.3 (9.0)
$\langle I/\sigma(I) \rangle$	15.0 (5.5)
$R_{\text{merge}}^\dagger$ (%)	11.1 (36.1)
$R_{\text{meas}}^\ddagger$ (%)	12.2 (40.5)
Overall B factor from Wilson plot (Å ²)	44.5

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of multiple observations. $^\ddagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the data multiplicity.

3. Results and discussion

His-tagged LsAspR was overexpressed in *E. coli* cells and purified to homogeneity. LsAspR crystals suitable for X-ray diffraction experiments were obtained using 25% (v/v) PEG MME 550, 5% (v/v) 2-propanol, 0.1 M sodium acetate pH 4.8. The crystal belonged to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 104.68$, $c = 97.29$ Å (Table 3). The diffraction data set was collected at 2.6 Å resolution. Considering the presence of two monomers in the asymmetric unit, the crystal volume per protein mass (V_M) is $2.82 \text{ Å}^3 \text{ Da}^{-1}$ and the solvent content is 56% (Matthews, 1968). These values are within the ranges frequently found in protein crystals. However, a map of the self-rotation function at 3.0 Å resolution showed no significant peaks in the $\chi = 180^\circ$ plane apart from those from crystallographic symmetries, although there are several weak peaks at the 2σ level. For an examination of the translational symmetry, the native Patterson function was calculated at 3.0 Å resolution, but the map showed no clear peaks apart from the origin peak. The results of both calculations did not provide reliable evidence that a dimer of two subunits related by molecular twofold symmetry exists in the asymmetric unit.

Molecular-replacement calculations were carried out using *MOLREP* (Vagin & Teplyakov, 2010) in space groups $P3_121$ and $P3_221$. The coordinates of PhAspR (PDB entry 1jfl; Liu *et al.*, 2002), which shares 31% amino-acid sequence identity with LsAspR, was selected as a starting model. The search model was prepared as a mixed model in which the side chains of nonconserved residues were truncated at the γ atom using *CHAINS*AW (Stein, 2008). Molecular replacement with the *CHAINS*AW-prepared model of chain *B* of PhAspR gave a solution in space group $P3_121$ as two independent chains. The R factor and the score were 0.590 and 0.284, respectively.

Inspection of the solution with *Coot* (Emsley *et al.*, 2010) revealed that the two chains form a dimer in the asymmetric unit and that the mode of dimer formation was quite similar to that of PhAspR. In addition, the formation of the dimer corresponded to the molecular weight of LsAspR of 52 kDa as estimated by gel-filtration chromatography (Oikawa *et al.*, manuscript in preparation). In this case, the number of subunits in the asymmetric unit was equivalent to the number of polypeptide chains estimated from the crystal data. For space group $P3_221$, a solution was obtained with one chain in the asymmetric unit. However, the symmetry-related chains did not form a dimer in the crystal. These results led to space group $P3_121$ for the present crystal.

The structural model obtained in space group $P3_121$ was subsequently subjected to rigid-body refinement followed by restrained refinement using *REFMAC5* (Murshudov *et al.*, 2011). Initial restrained refinement resulted in R and R_{free} values of 0.398 and 0.468, respectively. Subsequently, several rounds of model building and structure refinement were performed for this structural model of LsAspR. C^α -atom least-squares fitting of the two chains gave the rotation operation $(\omega, \varphi, \chi) = (64.6, 77.1, 178.6^\circ)$, which corresponded to one of the weak peak positions present in the self-rotation function. One possible reason why the self-rotation function did not clearly show the existence of twofold symmetry is the presence of higher average B factors for one of the two chains: 37 Å^2 for one chain and 26 Å^2 for the other in the current structural model. Therefore, the former chain may have more missing residues than the latter, which could lead to the ambiguous result of the self-rotation function. Further model building and structural refinement of LsAspR are currently in progress.

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