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Characterization of D-amino acid aminotransferase from *Lactobacillus salivarius*

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ABSTRACT

We searched a UniProt database of lactic acid bacteria in an effort to identify D-amino acid metabolizing enzymes other than alanine racemase. We found a D-amino acid aminotransferase (D-AAT) homologous gene (UniProt ID: Q1WRM6) in the genome of *Lactobacillus salivarius*. The gene was then expressed in *Escherichia coli*, and its product exhibited transaminase activity between D-alanine and α-ketoglutarate. This is the first characterization of a D-AAT from a lactic acid bacterium. *L. salivarius* D-AAT is a homodimer that uses pyridoxal-5′-phosphate (PLP) as a cofactor; it contains 0.91 molecules of PLP per subunit. Maximum activity was seen at a temperature of 60 °C and a pH of 6.0. However, the enzyme lost no activity when incubated for 30 min at 30 °C and pH 5.5 to 9.5, and retained half its activity when incubated at pH 4.5 or 11.0 under the same conditions. Double reciprocal plots of the initial velocity and D-alanine concentrations in the presence of several fixed concentrations of α-ketoglutarate gave a series of parallel lines, which is consistent with a Ping-Pong mechanism. The K_m values for D-alanine and α-ketoglutarate were 1.05 and 3.78 mM, respectively. With this enzyme, D-*allo*-isoleucine exhibited greater relative activity than D-alanine as the amino donor, while α-ketobutylate, glyoxylate and indole-3-pyruvate were all more preferable amino acceptors than α-ketoglutarate. The substrate specificity of *L. salivarius* D-AAT thus differs greatly from those of the other D-AATs so far reported.

1. Introduction

Nearly all proteins are constructed from 20 types of α-amino acids, all of which but glycine are optically active L-forms. Thus, L-α-amino acid exclusivity has been regarded as a central feature of biochemical and nutritional materials. On the other hand, recently developed analytical techniques have revealed the presence of D-amino acids, as the isomers of L-α-amino acids, in various organisms, including microorganisms, plants and fish [1,2]. In particular, numerous bacteria are known to produce specific D-amino acids such as D-alanine and D-glutamate [3,4], which are used for the synthesis of peptidoglycans, the main constituents of bacterial cell walls [5].

Bruckner et al. reported that a lactic acid bacterium, *Lactobacillus curvatus*, produces large amounts of D-alanine (100D/(D + L): 79%) [6]. In addition, we recently observed that many lactic acid bacteria produce high levels of D-alanine, D-aspartate and D-glutamate (unpublished data). However, little is known about the biosynthesis and degradation of D-amino acids, and the physiological significance of the enzymes catalyzing D-amino acid metabolism in lactic acid bacteria is also not well understood. Thus, characterization of the enzymes that catalyze D-amino acid metabolism in lactic acid bacteria remains an important goal.

In lactic acid bacteria, D-amino acid-related enzyme except for alanine, glutamate and aspartate racemases [7–11] has not been characterized to date. To further investigate D-amino acid metabolism in lactic acid bacteria, we searched for D-amino acid-related enzymes other than alanine racemase in the UniProt genome database of lactic acid bacteria. We found a hypothetical gene (UniProt ID: Q1WRM6) encoding a D-amino acid aminotransferase (D-AAT) that catalyzes transamination between D-amino acids, including D-alanine, and α-keto acids in *L. salivarius*. Up to now, nothing had been known about D-AATs in lactic acid bacteria. In this study, the hypothetical gene from *L. salivarius* was cloned and expressed in *Escherichia coli* and the characteristics of its product was investigated in order to gain information about the production of specific D-amino acids in lactic acid bacteria.

2. Experimental

2.1. Plasmid construction

The ORF coding for D-AAT in *L. salivarius*, Q1WRM6 in the UniProt database, was amplified using PCR with the chromosomal DNA from *L. salivarius* UCC118. The primer set used for the amplification was as follows: daat-f 5′-ATTTCATATG

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AAGCAAGTTGGATACTAC-3′ (*Nde* I site is underlined) and daat-r 5′-TTACTCGAGTTTAACGACTTACAGTTTCAG-3′ (*Xho* I site is underlined). The PCR products were digested with *Nde* I and *Xho* I and then ligated into pET 28a (Novagen, Darmstadt, Germany), which had been digested with the same restriction enzymes. The resultant plasmid, pDaat, was used for the expression of the D-AAT protein.

2.2. Enzyme expression and purification of recombinant protein

E. coli BL21-CodonPlusTM(DE3)-RIL cells (Stratagene, CA, USA) harboring pDaat were cultured for about 18 h at 25 °C with shaking (75 rpm). Using overnight expression medium (1 l), which contained 45 g/l Overnight Express™ Instant LB Medium (Novagen, Darmstadt, Germany) and 1% (v/v) glycerol, the enzyme was induced for about 18 h under the same conditions. The cells were then harvested by centrifugation (8500 × *g* for 10 min at 4 °C) and the cell pellet (wet weight: 1 g) was resuspended and mixed with 10 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 300 mM KCl, 10 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. This cell suspension was sonicated and centrifuged again as described above. The resultant supernatant was then applied to a Ni affinity column, and the eluate containing the purified enzyme was dialyzed against 100 volumes of 50 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 0.1 mM EDTA and 0.5 mM dithiothreitol for 12 h with two changes of the buffer solution at 4 °C. The dialysate was then concentrated by ultrafiltration using an Amicon Ultra (Merck Millipore, MS, USA). The resultant enzyme solution was added to an equal volume of glycerol solution and stored at −20 °C.

2.3. Enzyme assays

D-AAT activity was assessed by measuring the initial velocity of pyruvate formation from D-alanine using a lactate dehydrogenase (LDH)-coupled assay [12,13]. In addition, UPLC (ultra-performance liquid chromatography) analysis [14] and a modified salicylaldehyde method [15] were used when evaluating amino donor and acceptor specificity, respectively. The standard mixture used for the LDH-coupled reaction contained 100 mM potassium phosphate buffer (pH 7.5), 50 mM D-alanine, 20 mM α-ketoglutarate, 0.05 mM PLP, 0.1 mM NADH, 5 units/ml LDH (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and purified D-AAT in a total volume of 1 ml. Under such conditions, LDH activity is much higher than that of D-AAT. LDH was stored in 100 mM potassium phosphate buffer (pH 7.5) containing 50% (v/v) glycerol at −20 °C until use. The standard assay was run at 30 °C for 1 min. Reductions in NADH were monitored by measuring the decrease of absorbance at 340 nm using a spectrophotometer (V-630BIO, JASCO Corp., Tokyo, Japan) equipped with a thermostat (ETC-717, JASCO Corp., Tokyo, Japan). An extinction coefficient (ε) of 6220 M^{-1} cm^{-1} was used for NADH.

Amino donor specificity was determined using UPLC by measuring the rate of D-glutamate formation from α-ketoglutarate in the presence of various D-amino acids. The standard reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.5), 50 mM D-amino acid, 20 mM α-ketoglutarate, 0.05 mM pyridoxal-5′-phosphate (PLP) and the purified D-AAT. The enzyme reaction was run at 30 °C for 1 min and was stopped by adding 1 ml of 20% trichloroacetic acid. The reaction mixture was then incubated for 1 min on ice and 0.5 ml of 4 M NaOH was added to neutralize the mixture. UPLC analysis was performed using an ACQUITY UPLC TUV system consisting of a Waters Binary Solvent Manager, Sample Manager, FLR Detector and AccQ-Tag Ultra 2.1 × 100-mm column (Waters, Tokyo, Japan) with an eluent flow rate of 0.25 ml/min [14]. The column temperature was 30 °C, and the fluorescent wavelengths used for the FLR Detector were 350 and 450 nm. The eluent was linearly graduated using 85% 50 mM sodium acetate buffer (pH 5.9) and 15% acetonitrile.

Amino acceptor specificity was determined using a modification of the salicylaldehyde method by measuring the rate of pyruvate formation from D-alanine in the presence of an α-keto acid. The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 50 mM D-alanine, 20 mM α-keto acid, 0.05 mM pyridoxal-5′-phosphate and purified D-AAT. The reaction was run at 30 °C for 1 min in a total volume of 1 ml, and the reaction was stopped by adding 1 ml of 60% potassium hydroxide, after which 0.5 ml of 2% salicylaldehyde dissolved in 99% ethanol was added, and the mixture was incubated for 30 min at 30 °C. Thereafter, 1.5 ml of cold distilled water was added to the orange colored reaction mixture and absorbance at 480 nm was measured. The reaction mixture was also incubated for 1 min without the purified enzyme. The enzyme concentration was determined using the Bradford method with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, CA, USA). All enzyme assays were performed more than 3 times.

2.4. Effect of temperature on enzyme activity and stability

The effect of temperature on D-AAT activity was examined using the both standard reaction mixture for a LDH-coupled assay and D-glutamate assay method using UPLC at temperatures ranging from 30 °C to 70 °C. The thermostability of D-AAT was also examined using the same method. The purified enzyme was pre-incubated for 30 min at temperatures ranging from 30 °C to 70 °C.

2.5. Effect of pH on enzyme activity and stability

The effect of pH on D-AAT activity was examined by both the LDH-coupled assay and D-glutamate assay using UPLC with 100 mM acetate buffer (pH 4.0–5.5) or 100 mM potassium phosphate buffer (pH 5.5–8.0), instead of 100 mM potassium phosphate buffer used otherwise (pH 7.5). To assess the effect of pH on the stability of D-AAT, the same standard reaction was used with several buffers, including 100 mM acetate (pH 4.0–5.5), potassium phosphate (pH 5.5–8.0), Tris–HCl buffer (pH 8.0–9.0), carbonate (pH 9.0–10.5) and KH_2PO_4–NaOH (pH 10.5–12.0).

2.6. Effect of compounds on enzyme activity

Also examined were the effects on D-AAT activity of various compounds, including hydroxylamine, D-penicillamine, phenylhydrazine, *N*-ethylmaleimide, EDTA, L-alanine, L-glutamate, L-serine, L-cysteine, D-cysteine, NaCl, $MgCl_2$, KCl, $CaCl_2$, $MnCl_2$, $FeCl_2$, $CuCl_2$, $SrCl_2$ and $HgCl_2$. The activity after addition of a compound to the standard reaction mixture for the UPLC method was evaluated.

2.7. Kinetic analysis

Steady-state kinetic analyses of D-AAT were performed using several concentrations of D-alanine and α-ketoglutarate as substrates. Initial velocity was measured using the LDH coupling method at different substrate concentrations. The D-alanine concentrations used were 0.250, 0.333, 0.500, 1.00 and 2.00 mM, and the α-ketoglutarate concentrations were 0.500, 0.563, 0.625, 0.750 and 1.00 mM.

2.8. Molecular mass determination

The molecular mass of purified D-AAT was determined by gel filtration using a HiLoad 26/600 Superdex 200 pg column (GE Healthcare, Waukesha, WI, USA) with 10 mM potassium phosphate buffer (pH 7.5) containing 150 mM NaCl as the eluent at a flow rate of 0.5 ml/min. Bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B_{12}

(1.35 kDa) served as standards to build the calibration curve. In addition, subunit molecular weight was determined by SDS-PAGE using 12.5% polyacrylamide gel and precision Plus Proteins Standards (Bio-Rad, CA, USA) were used.

2.9. Determination of PLP content

The PLP content of the purified enzyme was determined using the method of Peterson and Sober [16]. Briefly, the purified enzyme (1.11 mg/ml) was dialyzed against 100 mM of potassium phosphate buffer (pH 7.5). The dialyzed enzyme was then incubated with 0.1 M NaOH at 30 °C for 5 min, and the absorbance at 388 nm was measured. The PLP content was calculated using the extinction coefficient for PLP in 0.1 M NaOH ($\varepsilon = 6600\ M^{-1}\ cm^{-1}$) [17].

2.10. Spectral analysis

For spectral analysis, enzyme solution (0.936 mg/ml) was dialyzed against 100 volumes of 50 mM potassium phosphate buffer (pH 7.0) for 12 h with two changes of the buffer solution. The absorption spectrum of the D-AAT solution was then measured using a spectrophotometer (V-630BIO, JASCO Corp., Tokyo, Japan). In some cases, the D-AAT was reduced with 50 μM sodium borohydride in 50 mM potassium phosphate buffer (pH 7.0), after which the solution was dialyzed against 50 mM potassium phosphate buffer, and the absorption spectrum of the reduced enzyme was measured.

2.11. Sequence analysis of D-AAT

The primary amino acid sequence of D-AAT from *L. salivarius* was analyzed and compared with those of other D-AATs so far reported [12,18,19]. The amino acid sequences were retrieved from the UniProt database (http://www.uniprot.org/), and multiple alignments were performed using TCoffee (http://www.tcoffee.org/Projects/tcoffee/).

3. Results and discussion

3.1. Purification of L. salivarius D-AAT from recombinant E. coli

We screened a database for genes having sequence homology with D-AAT and found an *L. salivarius* gene (UniProt ID: Q1WRM6) that showed 35% sequence homology with D-AAT from *Bacillus* sp. However, no similar gene was found in any of the other 24 species of lactic acid bacteria listed in the database. We cloned the Q1WRM6 gene and obtained recombinant *E. coli* BL21 cells containing a hybrid plasmids harboring the Q1WRM6 gene. Moreover, prepared cell extracts contained high levels of D-AAT activity (0.713 units/mg). The high activity levels indicated that *L. salivarius* D-AAT was being overexpressed as a recombinant protein, since the host *E. coli* BL21 has no intrinsic D-AAT activity. The overexpressed enzyme was then purified to homogeneity (Fig. 1) on Ni-NTA Agarose (QIAGEN, Venlo, Netherlands). Purification of the enzyme was achieved using a single chromatography step. The final yield was 28.1% (Table 1).

3.2. Effect of temperature on enzyme activity and stability

We first examined the effect of temperatures ranging from 30 °C and 70 °C on the activity of the purified D-AAT. The *L. salivarius* enzyme showed maximum activity at 60 °C (Fig. 2A), which is similar to D-AATs from *Bacillus* and *Geobacillus* sp. Examination of the effect of temperature on enzyme stability showed that there was no loss of activity until the temperature reacted 45 °C, but there was a steep decline in activity at temperatures above 50 °C (Fig. 2B).

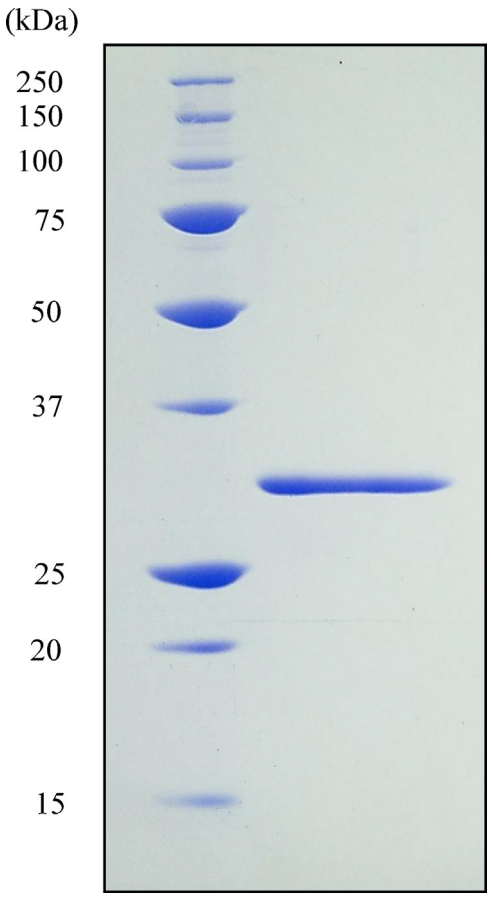

Fig. 1. SDS-PAGE of purified *L. salivarius* D-AAT. The left and right lanes show molecular mass markers and the D-AAT elution pattern, respectively.

Thus *L. salivarius* D-AAT shows less thermostability than D-AATs from *Bacillus* [12,19] and *Geobacillus* sp. [18], which show no loss activity below 60 °C.

3.3. Effect of pH on enzyme activity and stability

We examined the effect of pH on this enzyme's activity at pHs between 4.0 and 8.0 (Fig. 3A). The *L. salivarius* D-AAT showed maximum activity at around pH 6.0. This is much different from the *Bacillus* and *Geobacillus* sp. enzymes, which exhibit maximum activity at alkaline pHs, ranging from pH 8 to 9 [12,19] and very little activity (about 10% of maximum) at pH 6.0 [18]. In addition, *L. salivarius* D-AAT was stable over a wide pH range, from 5.5 to 9.5 (Fig. 3B), and it retained half its activity after incubation for 30 min at 30 °C and pH 4.5 or 11.0. By contrast, *Geobacillus* sp. D-AAT was completely inactive after incubation at pH 5.5 under the same conditions [18]. These results show that *L. salivarius* D-AAT is stable and functions well under acidic conditions, which makes it different from the *Bacillus* and *Geobacillus* enzymes.

3.4. Substrate specificity

The amino donor specificity of *L. salivarius* D-AAT was evaluated using various amino acids in the presence of α-ketoglutarate as the amino acceptor. *L. salivarius* D-AAT showed broad amino donor specificity, and high activity was observed with several D-amino acids, including D-*allo*-isoleucine, D-α-aminobutyrate and D-methionine, in addition to D-alanine (Table 2). D-*allo*-isoleucine was the most preferable amino donor, but interestingly D-isoleucine is totally inert. In addition, this enzyme also showed

Table 1
Purification of *L. salivarius* D-AAT from *E. coli* BL 21.

Step[a]	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	247	176	0.713	100	1.00
Ni-NTA Agarose	2.00	49.5	24.7	0.809	34.3

[a] Wet weight of cells was 2.62 g.

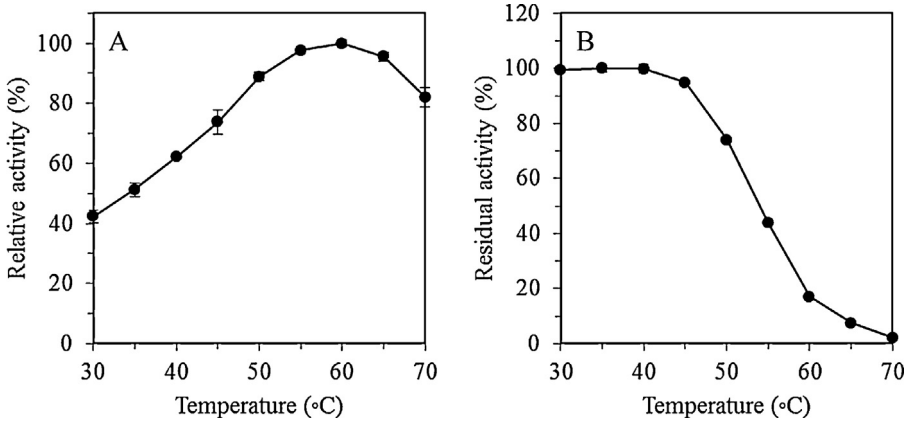

Fig. 2. Effect of temperature on enzyme activity (a) and stability (b). (a) The enzyme was assayed at the indicated temperatures. The maximum rate was 59.6 ± 0.613 μmol/min/mg (n = 3). (b) The enzyme was incubated for 30 min at pH 7.5 and the indicated temperatures (n = 3). Activity was then assayed under standard assay conditions (30 °C and pH 7.5). The maximum rate was 26.6 ± 0.272 μmol/min/mg.

very broad amino acceptor specificity among α-keto acids (Table 3). α-Ketobutyrate, glyoxylate, indole-3-pyruvate, α-ketovalerate, 3-methyl-2-ketobutyrate and 4-hydroxyphenyl-pyruvate all supported higher activity as the amino acceptor than α-ketoglutarate or pyruvate, with α-ketobutyrate being the most active of the α-keto acids. It thus appears the amino donor specificity of *L. salivarius* D-AAT differs substantially from those of other D-AATs. With respect to D-AATs from *Bacillus* sp., for example, branched chain amino acids are known to be inert or to support extremely low activity as the amino donor [12,19]. In addition, the observation that the amino acceptor activities of α-ketobutyrate and glyoxylate are much greater than that of α-ketoglutarate sets *L. salivarius* D-AAT well apart from the *Bacillus* sp. strain YM1, *Bacillus sphaericus* and *Geobacillus toebii* SK1 enzymes [18]. In the case of *Bacillus* sp. D-AAT, for example, the activities of the amino acceptors α-ketobutyrate (100%) and glyoxylate (30%), relative to α-ketoglutarate, are much lower than the relative activities obtained with the *L. salivarius* enzyme (401% and 222%, respectively).

3.5. Kinetic analysis of D-AAT

When D-AAT activities were measured at several concentrations of D-alanine and α-ketoglutarate, the Lineweaver–Burk plots for the different concentrations of D-alanine and α-ketoglutarate gave series of parallel lines (Fig. 4A and C). From secondary plots, the K_m values for D-alanine and α-ketoglutarate were calculated to be 1.05 and 3.78 mM, respectively. The K_m value for D-alanine with *L. salivarius* D-AAT was lower than that (4.2 mM) with the *B. sphaericus* enzyme [20] and was compatible to the value obtained (1.4 mM) with the *Geobacillus* sp. strain KLS1 enzyme [18]. On the other hand, the K_m value (3.78 mM) for α-ketoglutarate with *L. salivarius* D-AAT was fairly high, as compared to those with the *Bacillus* and *Geobacillus* enzymes [18,20].

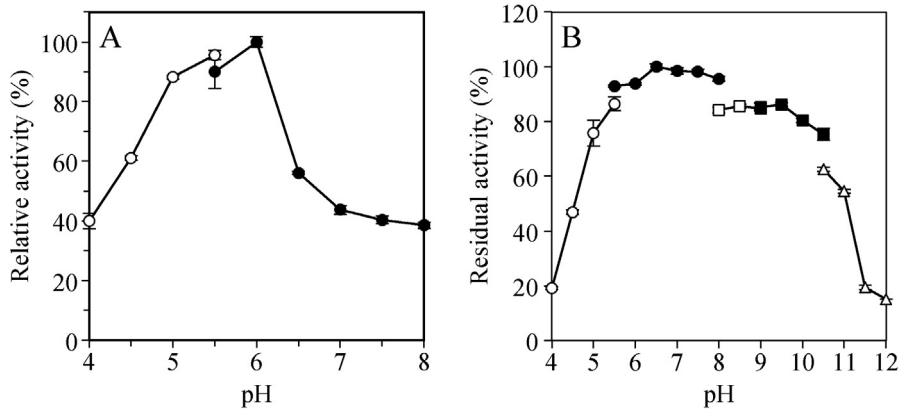

Fig. 3. Effect of pH on enzyme activity (a) and stability (b). (a) The enzyme was incubated for 1 min at 30 °C and at the indicated pHs. The maximum rate was 65.4 ± 1.14 μmol/min/mg (n = 3). (b) The enzyme was incubated for 30 min at 30 °C and the indicated pHs. Activity was then assayed at under the standard conditions. The maximum rate was 45.3 ± 0.423 μmol/min/mg (n = 3). Open circles: acetate buffer, filled circles: potassium phosphate buffer, open squares: Tris–HCl buffer, filled squares: carbonate buffer, open triangles: KH_2PO_4–NaOH buffer.

Table 2
Amino donor specificity of D-AAT.

Substrate	Relative activity (%)			
	L. salivarius	*Bacillus* sp. YM-1 [12]	*B. sphaericus* [19]	*G. toebii* SK1 [18]
D-Alanine	100	100	100	100
D-*allo*-Isoleucine	104 ± 14.9	–	0	–
D-α-Amino butyrate	89.7 ± 2.09	98	97	89
D-Methionine	89.2 ± 2.99	19	61	0.7
D-Leucine	83.5 ± 3.58	2.4	8	–
D-Norvaline	82.6 ± 8.90	34	83	8.2
D-Valine	83.0 ± 1.75	3.2	0	1.9
D-Histidine	75.1 ± 6.36	1.9	6	1.9
D-Norleucine	70.8 ± 2.14	5	46	–
D-Pyroglutamate	41.4 ± 2.40	–	–	–
D-Threonine	12.5 ± 0.59	0	0	0.9
D-Arginine	5.13 ± 0.69	1	6	–
D-Serine	0	60	30	70
D-Cystein	0	25	31	11
D-Proline	0	1.2	0	12
D-Asparagine	0	0.9	32	–
D-Isoleucine	0	0.2	0	–
D-*allo*-Threonine	0	0.1	0	–
D-Phenylalanine	0	0	0	–
β-Alanine	0	0	0	–
D-Aspartate	0	0	0	–
Glycine	0	–	–	–

The activities were assayed at 30 °C and pH 7.5 for 1 min. The 100% relative activity was 24.1 ± 1.31 μmol/min/mg. α-Ketoglutarate was used as the amino acceptor.

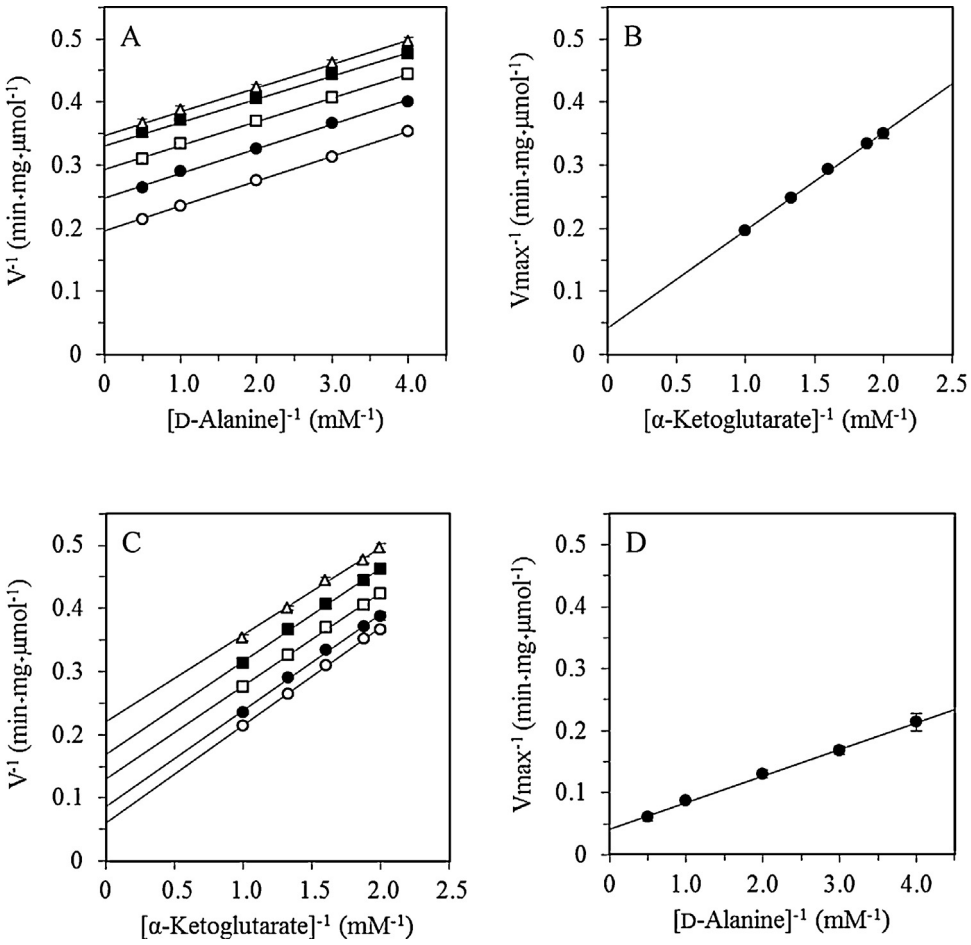

Fig. 4. Double reciprocal and secondary plots. The enzyme was incubated for 1 min at 30 °C and pH 7.5 in various concentrations of D-alanine and α-ketoglutarate. (a) Double reciprocal plots of initial velocity against the D-alanine concentration at several fixed concentrations of α-ketoglutarate (open circles: 1.00 mM, filled circles: 0.750 mM, open squares: 0.625 mM, filled squares: 0.536 mM, open triangles: 0.500 mM) ($n = 3$). (b) Secondary plots between the intercepts on the vertical axis in (a) and the reciprocals of the α-ketoglutarate concentrations. (c) Double reciprocal plots of the initial velocity against the α-ketoglutarate concentration at several fixed concentrations of D-alanine (open circles: 2.00 mM, filled circles: 1.00 mM, open squares: 0.500 mM, filled squares: 0.333 mM, open triangles: 0.250 mM) ($n = 3$). (d) Secondary plots between the intercepts on the vertical axis in (c) and the reciprocals of the D-alanine concentrations.

Table 3
Amino acceptor specificity of D-AAT.

Substrate	Relative activity (%)			
	L. salivarius	*Bacillus* sp. YM-1 [12]	*B. sphaericus* [19]	*G. toebii* SK1 [18]
α-Ketoglutarate	100	100	100	100
α-Ketobutyrate	401 ± 13.7	100	144	110
Glyoxylate	222 ± 13.8	34	24	–
Indole-3-pyruvate	203 ± 7.59	–	–	0
α-Ketovalerate	103 ± 1.65	88	70	–
3-Methyl-2-ketobutyrate	102 ± 2.21	–	–	–
4-Hydroxyphenylpyruvate	101 ± 1.21	–	–	–
Pyruvate[a]	98.1 ± 7.87	–	–	–
Oxaloacetate	91.1 ± 5.65	57	20	–
α-Ketohexanoate	89.5 ± 1.43	61	14	–
Phenylpyruvate	45.4 ± 1.66	12	2.3	2
α-Ketooctanoate	38.9 ± 1.51	–	–	–
α-Ketogluconate	26.1 ± 4.36	–	–	–
β-Fluoropyruvate	13.8 ± 0.27	–	–	–
β-Hydroxypyruvate	0	–	–	–

The activities were assayed at 30 °C and pH 7.5 for 1 min. The 100% relative activity was 24.1 ± 1.31 μmol/min/mg.
[a] When pyruvate was used as the amino acceptor, 50 mM D-glutamate was used as the amino donor.

3.6. Molecular mass determination

The gel filtration chromatography elution profile for *L. salivarius* D-AAT had a single peak corresponding to a molecular mass of about 56.5 kDa. In addition, SDS-PAGE (Fig. 1) gave one band corresponding to a molecular weight of 31.5 kDa. This indicates that *L. salivarius* D-AAT functions as a homodimer, like the other known D-AATs [12,18,19].

3.7. Effect of inhibitors and identification of PLP as a cofactor

We also examined the effect of various compounds on the activity of *L. salivarius* D-AAT (0.037 mg/ml). We found that hydroxylamine (0.10 mM), D-penicillamine (1 mM) and phenylhydrazine (1 mM) which are specific inhibitors of PLP-dependent enzymes, markedly inhibited the activity of this enzyme (Table 4). This suggests that PLP is an active cofactor with *L. salivarius* D-AAT, as it is with D-AATs from other microorganisms. In addition, *L. salivarius* D-AAT was markedly inhibited by $HgCl_2$, *N*-ethylmaleimide and *p*-chloromercuribenzoate (PCMB) which are typical SH inhibitors. Metallic salts also inhibited activity of *L. salivarius* D-AAT. In case of some D- and L-amino acids, L-alanine, L-glutamate and L-serine slight inhibited this enzyme activity. However L- and D-cysteine markedly or completely inhibited this enzyme activity.

To confirm that PLP serves as a cofactor with *L. salivarius* D-AAT, we measured the absorption spectrum of the enzyme at wavelengths ranging from 250 nm to 500 nm. We detected two peaks, at 280 nm and 420 nm, respectively (Fig. 5). Furthermore, upon addition of sodium borohydride, the 420 nm peak disappeared with the concomitant appearance of an absorption peak at around 325 nm (Fig. 5). These features of the enzyme's absorption spectrum indicate that PLP binds to the enzyme through Schiff-base formation with the active lysine residue and is fixed to the enzyme by borohydride-mediated reduction [19]. The PLP content of the enzyme was determined to be 0.91 mol of PLP/mol of enzyme subunit using the Peterson and Sober method [16].

3.8. Sequence analysis of L. salivarius D-AAT

The amino acid sequence of *L. salivarius* D-AAT was aligned and compared with the sequences of D-AATs from other sources (Fig. 6). The *L. salivarius* enzyme showed sequence homology with

Table 4
Effect of inhibitors on *L. salivarius* D-AAT.

Additive compound	Concentration (mM)	Relative activity
None		100
L-Ala	5.0	81.5 ± 2.38
L-Glu	5.0	94.2 ± 4.78
L-Ser	5.0	93.3 ± 5.97
L-Cys	5.0	50.0 ± 0.63
D-Cys	5.0	0
NaCl	1.0	74.3 ± 7.97
$MgCl_2$	1.0	78.1 ± 3.52
KCl	1.0	91.4 ± 3.50
$CaCl_2$	1.0	78.2 ± 1.91
$MnCl_2$	1.0	69.3 ± 1.15
$FeCl_2$	1.0	69.8 ± 0.14
$CuCl_2$	1.0	68.9 ± 1.59
$SrCl_2$	1.0	81.7 ± 4.07
Hydroxylamine	0.1	44.9 ± 6.33
Phenylhydrazine	1.0	0
D-Penicillamine	1.0	0
N-Ethylmaleimide	1.0	67.3 ± 6.09
$HgCl_2$	0.1	0
PCMB	0.1	0
EDTA	5.0	116 ± 3.93

The activities were assayed at 30 °C and pH 7.5 for 1 min. The 100% relative activity was 24.1 ± 1.31 μmol/min/mg.

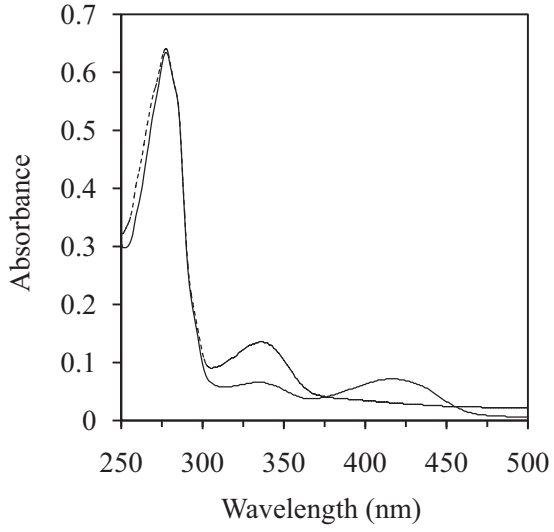

Fig. 5. Absorption spectra of the enzyme. The spectra of the native enzyme (solid line) and the reduced enzyme in the presence of 50 μM sodium borohydride (broken line) were measured at 30 °C and pH 7.5. The enzyme concentration was 0.936 mg/ml.

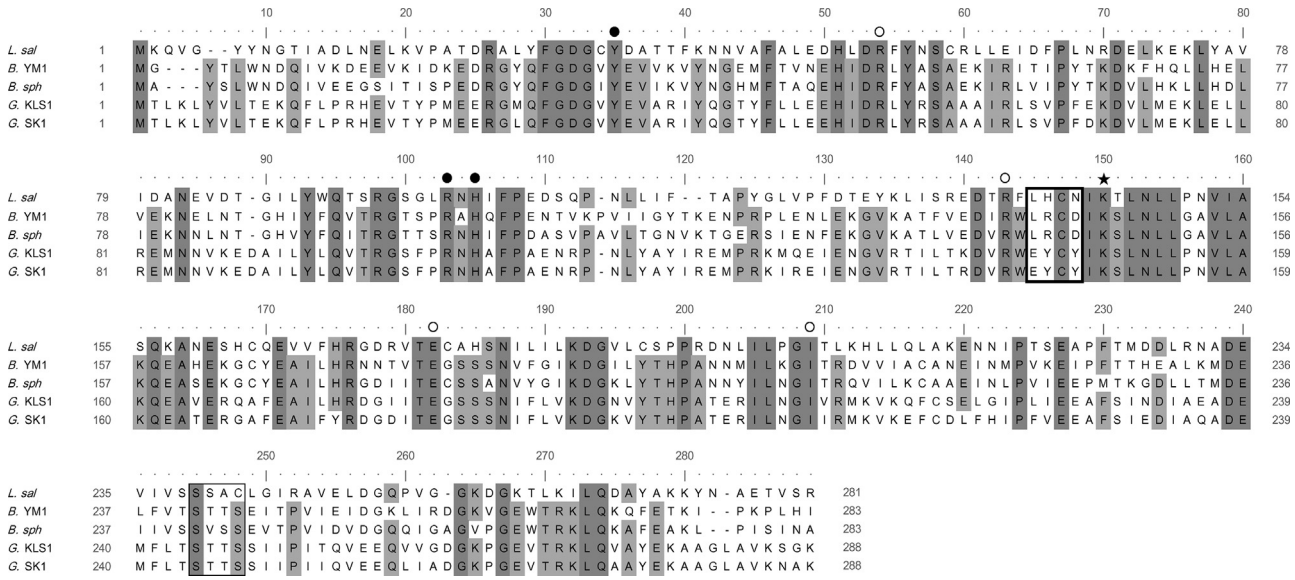

Fig. 6. Amino acid sequence alignment of *L. salivarius* D-AAT with other D-AATs: *L. sal*: *L. salivarius*, *B.* YM1: *Bacillus* sp. strain YM1, *B. sph*: *B. sphaericus*, *G.* KLS1: *Geobacillus* sp. strain KLS1, and *G.* SK1: *G. toebii* SK1. The catalytic lysine, substrate-binding [Y32 (Y33), R99 (R100) and H101 (H102)] and cofactor-binding residues [R51 (R52), E178 (E176) R139 (R137) and I205 (I203)] are marked by filled star, filled circles and open circles respectively. The loops from S239 to C242 and 139-LHCN-142 sequences are surrounded in a line and bold lines respectively.

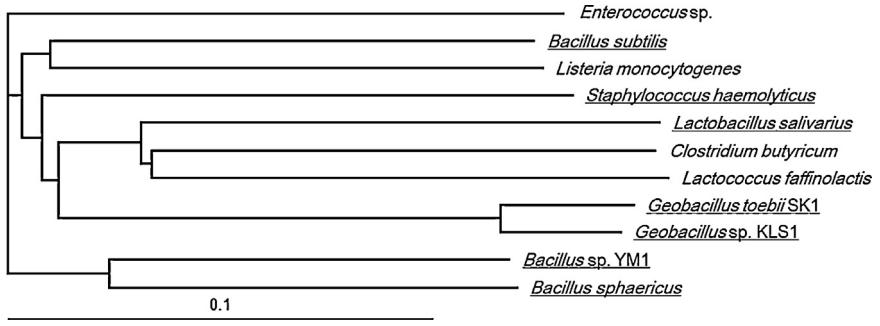

Fig. 7. Phylogenetic tree of *L. salivarius* D-AAT with other D-AATs. Bacterial D-AATs whose enzymatic activity has been experimentally characterized are underlined.

those from *B. sphaericus* (38% homology), *Bacillus* sp. YM-1 (35%), *Geobacillus* sp. KLS1 (35%) and *G. toebii* SK1 (33%). Notably, higher sequence homology (about 45%) was observed between *Bacillus* D-AATs and *Geobacillus* enzyme. In addition, the phylogenetic tree showed that *L. salivarius* D-AAT is clustered away from the *Bacillus* and *Geobacillus* species D-AATs (Fig. 7). This suggests that, with respect to their overall sequences, *L. salivarius* D-AAT differs substantially from the *Bacillus* and *Geobacillus* species enzymes.

Nonetheless, when we compared the residues responsible for enzyme function and substrate and PLP binding between the *Bacillus* and *Geobacillus* species D-AATs and *L. salivarius* D-AAT, we found that the catalytic K146 of *Bacillus* sp. YM1 D-AAT (K144 in *L. salivarius* D-AAT), the carboxylate traps that define the position and orientation of the substrate-binding [Y32 (Y33), R99 (R100) and H101 (H102)], and the cofactor-binding residues [R51 (R52), E178 (E176) R139 (R137) and I205 (I203)] are well conserved. On the other hand, the loop from S241 to S244 (STTS), thought to be important for substrate specificity, is not conserved in *L. salivarius* D-AAT (S239 to C242, SSAC) [18,21]. Nor is the stable salt bridge formed by the R-D residues in the 141-LRCD-144 sequence of *Bacillus* sp. YM1 D-AAT [21] or the π/π interaction formed by the Y-Y residues in 144-EYCY-147 sequence of the *Geobacillus* enzyme [22]. In *L. salivarius* D-AAT, the corresponding sequence is 139-LHCN-142, and the H-N residues are clearly different from the two aforementioned sequences (R-D and Y-Y residues). This also suggests the functional properties of *L. salivarius* D-AAT differ from those of the *Bacillus* and *Geobacillus* D-AATs. Analyses of the 3D-structure and regulatory mechanisms governing *L. salivarius* D-AAT activity will be needed to fully understand this enzyme's physiological function. Those studies are now ongoing.

4. Conclusion

In this study, a D-AAT gene homolog was found in a lactic acid bacterium, *L. salivarius*. The gene was successfully expressed in an *E. coli* transformant, and the product showed strong D-AAT activity. A rapid, one-step purification of the enzyme was achieved, and the enzyme was characterized as the first D-AAT from a lactic acid bacterium. This D-AAT showed maximum activities at 60 °C and pH 6.0, and was highly stable and active under acidic conditions, which sets it apart from D-AATs from *Bacillus* and *Geobacillus* species. In addition, the substrate specificity of *L. salivarius* D-AAT differed from those of the *Bacillus* and *Geobacillus* enzymes. While D-branched chain amino acids, such as D-*allo*-Ile, D-Leu and D-Val, functioned well as amino donors with *L. salivarius* D-AAT, but were inert with the *Bacillus* and *Geobacillus* enzymes. Furthermore, a wide variety of α-keto acids functioned well as amino acceptors. α-Ketobutyrate, glyoxylate and indole-3-pyruvate showed 2–4 times greater reactivity than α-ketoglutarate as the amino acceptor. Thus, *L. salivarius* D-AAT appears to exhibit unique characteristics among D-AATs.

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