Biosci. Biotechnol. Biochem., **72** (5), 1299–1306, 2008

Glutamate Decarboxylase from *Lactobacillus brevis*: Activation by Ammonium Sulfate

Kazumi HIRAGA,[1] Yoshie UENO,[1,2] and Kohei ODA[1,†]

[1]*Department of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Goshokaido-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan*
[2]*Kyoto Prefectural Technology Center for Small and Medium Enterprise, 134 Chudoji, Minamimachi, Shimogyo-ku, Kyoto 600-8813, Japan*

Received December 4, 2007; Accepted January 13, 2008; Online Publication, May 7, 2008
[doi:10.1271/bbb.70782]

In this study, the glutamate decarboxylase (GAD) gene from *Lactobacillus brevis* IFO12005 (*Biosci. Biotechnol. Biochem.*, 61, 1168–1171 (1997)), was cloned and expressed. The deduced amino acid sequence showed 99.6% and 53.1% identity with GAD of *L. brevis* ATCC367 and *L. lactis* respectively. The His-tagged recombinant GAD showed an optimum pH of 4.5–5.0, and 54 kDa on SDS–PAGE. The GAD activity and stability was significantly dependent on the ammonium sulfate concentration, as observed in authentic GAD. Gel filtration showed that the inactive form of the GAD was a dimer. In contrast, the ammonium sulfate-activated form was a tetramer. CD spectral analyses at pH 5.5 revealed that the structures of the tetramer and the dimer were similar. Treatment of the GAD with high concentrations of ammonium sulfate and subsequent dilution with sodium glutamate was essential for tetramer formation and its activation. Thus the biochemical properties of the GAD from *L. brevis* IFO12005 were significantly different from those from other sources.

Key words: ammonium sulfate; glutamate decarboxylase (GAD); gamma-aminobutyric acid (GABA); *Lactobacillus brevis*

Glutamate decarboxylase (GAD) is a pyridoxal 5′-phosphate (PLP) dependent enzyme that catalyzes the irreversible alpha-decarboxylation of L-glutamate to gamma-aminobutyric acid (GABA). GAD is widely distributed in nature, in microorganisms, plants, and animals.[1)] GABA is an amino acid not found in proteins, a major inhibitory neurotransmitter with hypotensive and diuretic effects in animals.[2,3)] Some GABA-containing foods, such as tea,[4)] beni-koji,[5)] and germinated brown rice[6)] have been developed.

Enzymatic properties and molecular cloning of GAD genes in *Escherichia coli gad* A (Gad A), *gad* B (Gad B),[7–10)] *Lactococcus lactis gad* B (Gad B),[11–15)] *Neurospora crassa* (Gad protein),[16)] *Listeria monocytogenes gad* A (Gad A), *gad* B (Gad B),[17)] *Shigella flexneri gad* A (Gad A), *gad* B (Gad B),[18)] and *Aspergillus oryzae gad* A (Gad A)[19,20)] have been reported so far, but only a few reports on the biochemical properties of GAD have been published, due to its intrinsically unstable nature.

Among these, *E. coli* GADs (two isoforms, Gad A and Gad B) have been characterized in detail. One of the unique points is that activity was stimulated by NaCl.[21,22)] The GADs were shown to prevent acidification of culture medium by converting glutamic acid to GABA (an acid-tolerance mechanism).[23)] The crystal structures of GADs have been elucidated.[22,24)]

We have screened for GABA-producing microorganisms from fermented foods and other sources, and have succeeded in isolating several lactic acid bacteria.[25)] In parallel with this screening, we selected *Lactobacillus brevis* IFO12005 as a standard strain in order to compare with the biochemical properties of GAD from other sources, but the productivity of GAD was not sufficient to study biochemical properties. In addition, GAD was also unstable during purification by several column chromatographies. Hence we carried out cloning of the GAD gene and its expression using *E. coli* in order to get large amounts of GAD. Using the recombinant GAD, we elucidated some unique characteristics of the GAD as activation by ammonium sulfate.

Quite recently, gene cloning and expression of GAD from *Lactobacillus brevis* OPK-3 in *E. coli* was reported,[26)] but the recombinant GAD was not purified and characterized. One of the reasons why that team did not study the biochemical properties of a purified enzyme might be the unstable nature mentioned above.

† To whom correspondence should be addressed. Fax: +81-75-724-7710; E-mail: bika@kit.ac.jp
Abbreviations: GAD, glutamate decarboxylase; GABA, gamma-aminobutyric acid; PLP, pyridoxal 5′-phosphate

Materials and Methods

Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Tokyo), New England Biolabs (Ipswich, MA), and Toyobo (Osaka, Japan). A DIG DNA labeling kit was purchased from Roche Diagnostics (Basel, Switzerland). A nylon membrane was from Pall (East Hills, NY) and a PVDF membrane was from Millipore (Billerica, MA). Ni-NTA agarose and pQE-70 were from Qiagen (Hilden, Germany). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Strains and culture conditions. Lactobacillus brevis IFO 12005 was cultivated under static conditions in glucose-yeast extract-peptone (GYP) medium containing 1% sodium glutamate for 24 h at 30 °C. *E. coli* JM109 harboring an expression plasmid was cultivated in LB broth containing 50 μg/ml of ampicillin at 37 °C for 24 h. *E. coli* Rosetta-gami B (DE3) pLac I harboring an expression plasmid was cultivated under similar conditions with 50 μg/ml of ampicillin and 15 μg/ml of kanamycin.

GAD gene cloning. Genomic DNA was purified from *L. brevis* IFO 12005 using the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI), and was subjected for PCR. A partial GAD gene was amplified by PCR using KOD-plus (Toyobo) and the following primers: 5′-CAR ATG GAR CCN CAR GCN GAY GA-3′ and 5′-GG RTA NGC NGG NAC YTG CCA-3′. The amplified DNA fragment was subcloned into pPCR-Script Amp predigested cloning vector with a PCR-Script Amp cloning kit (Stratagene, La Jolla, CA). The plasmid obtained was digested with *Eco* RI, and the insert DNA (about 1 kbp) was labeled with a DIG-labeling kit (Roche Diagnostics) and used as a probe in screening.

A *Pst* I/*Sal* I digested genomic DNA fragment of *L. brevis* IFO 12005 was ligated into the *Pst* I/*Sal* I site of pUC18, then transformed into *E. coli* JM109 to construct a partial genomic DNA library. About 500 colonies were screened by colony hybridization using a DIG-labeled DNA probe. A 5-kbp DNA fragment was sequenced by gene walking. A Taq dye deoxy™ terminator cycle sequencing kit (Perkin Elmer) and Perkin Elmer model 373A DNA sequencer were used in sequencing.

Construction of expression plasmid. An expression vector, pQE-70, was initially digested with *Sph* I, blunt-ended with a DNA-blunting kit (Takara, Kyoto, Japan), and digested with *Bgl* II. A GAD gene of *L. brevis* IFO 12005 was amplified by PCR using the following primers: 5′-C ATG ATG AAT AAA AAC GAT CAG GAA AC-3′ and 5′-GAAGATCT AAC GGT GGT CTT GTT ATC TTG-3′. The DNA fragment obtained was digested with *Bgl* II, and after treatment with T4 polynucleotide kinase (Takara) it was ligated into pQE-70 vector, prepared as described above, to express a C-terminally $(His)_6$-tagged fusion protein in *E. coli*.

Purification of GAD-$(His)_6$ fusion protein. E. coli Rosetta-gami B (DE3) pLac I cells harboring the expression plasmid were shaken at 110 strokes/min overnight at 37 °C in LB medium containing 50 μg/ml of ampicillin and 15 μg/ml of kanamycin. The overnight culture was inoculated into the same medium. The cultivation was continued with shaking at 25 °C. The fusion protein was induced by the addition of 1 mM IPTG when the absorbance at 660 nm reached 0.6, and then it was cultured overnight at 25 °C. The cells, harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C, were washed once with chilled distilled water, and then suspended in phosphate-buffered saline (PBS) containing protease inhibitor cocktails (Roche Diagnostics). The cells were disrupted by ultrasonication at 4 °C, and then triton X-100 was added (final concentration, 1%), then it was left on ice for 1 h. The supernatant obtained after centrifugation at 15,000 × *g* for 20 min at 4 °C was applied to the Ni-NTA-agarose column. The column was washed with PBS, and then the fusion protein was eluted stepwise with 20 to 250 mM imidazole-containing PBS buffer.

Enzyme assay. Enzyme solution (0.1 ml) and 0.1 ml of 4 M ammonium sulfate were mixed. After incubation for several min at room temperature, 1.3 ml of substrate solution (20 mM sodium glutamate, 0.2 mM PLP, 0.2 M pyridine-HCl, pH 4.6) were added to the enzyme solution, and then the reaction mixture was incubated at 37 °C for 60 min. The reaction was stopped by boiling for 5 min, and GABA was analyzed by TLC (Merck, Darmstadt, Germany; solvent, n-butanol:acetic acid: water 3:2:1 (v/v/v); detection, ninhydrin), and quantified with an amino acid analyzer (Shimazdu LC-9A, using a Shim-pack Isc-07 Na column, Shimazdu, Kyoto, Japan).

One unit of enzyme activity (U) was defined as the amount of enzyme forming 1 micromole of GABA per min.

CD spectrum. The CD spectrum (200–250 nm) of the purified GAD (dissolved in 10 mM sodium McIlvain buffer, pH 5.5 or 7.0) was measured with a Jasco J-720-A spectropolarimeter (Nihon Bunko, Tokyo) at 25 °C using a quartz cuvette with a 0.1-cm path.

Gel-filtration column chromatography. A Superdex 200 10/300 GL (GE Healthcare, Uppsala, Sweden) size-exclusion column was equilibrated with buffer (50 mM sodium acetate buffer, pH 5.5, 0.5 M ammonium sulfate, 0.2 mM PLP, and 1 mM sodium glutamate) and calibrated with standards (1.35–67 kDa; Bio-Rad, Hercules, CA)

```
GAAAAATGATTAAGGAGGCAAGCCATATCGCAATACGGATATGACGACTATATGATGAAT      60
            rbs                                          M  M  N       3
AAAAACGATCAGGAAACACAGCAGATGATTAATAATGTGGATTTAGAAAAAACGTTTTTA     120
 K  N  D  Q  E  T  Q  Q  M  I  N  N  V  D  L  E  K  T  F  L      23
GGCAGTGTCGAAGCCGGGCAATCCTTACCCACCTATACATTACCAGATGATCCCATGGCA     180
 G  S  V  E  A  G  Q  S  L  P  T  Y  T  L  P  D  D  P  M  A      43
CCGGATGTTGCCGCTCAATTGGTGGAACACTATCGTTTAAATGAAGCCAAGGCTAATCAA     240
 P  D  V  A  A  Q  L  V  E  H  Y  R  L  N  E  A  K  A  N  Q      63
AACCTAGCGACCTTCTGTACCACGCAAATGGAACCACAAGCCGATGAATTAATGAAGAAC     300
 N  L  A  T  F  C  T  T  Q  M  E  P  Q  A  D  E  L  M  K  N      83
GCGTTGAATACCAATGCGATTGATAAATCGGAATACCCTAAGACCGCGGCAATGGAAAAT     360
 A  L  N  T  N  A  I  D  K  S  E  Y  P  K  T  A  A  M  E  N     103
TACTGTGTCAGCATGATTGCTCACCTATGGGGAATTCCTGACAATGAAAAGATTTACGAT     420
 Y  C  V  S  M  I  A  H  L  W  G  I  P  D  N  E  K  I  Y  D     123
GATTTCATTGGGACCTCAACTGTAGGTTCTTCTGAAGGATGTATGTTAGGCGGCTTGGCG     480
 D  F  I  G  T  S  T  V  G  S  S  E  G  C  M  L  G  G  L  A     143
CTACTACATAGTTGGAAGCACCGGGCCAAGGCAGCTGGTTTTGATATTGAAGACCTGCAT     540
 L  L  H  S  W  K  H  R  A  K  A  A  G  F  D  I  E  D  L  H     163
AGCCACAAGCCCAACTTGGTCATCATGTCAGGTTACCAAGTTGTTTGGGAAAAGTTCTGT     600
 S  H  K  P  N  L  V  I  M  S  G  Y  Q  V  V  W  E  K  F  C     183
ACCTATTGGAATGTCGAGATGCGCCAAGTGCCAATTAATGGTGACCAAGTTTCCTTAGAT     660
 T  Y  W  N  V  E  M  R  Q  V  P  I  N  G  D  Q  V  S  L  D     203
ATGGATCATGTGATGGATTATGTTGATGAAAATACGATTGGGATTATCGGAATTGAGGGC     720
 M  D  H  V  M  D  Y  V  D  E  N  T  I  G  I  I  G  I  E  G     223
ATTACGTACACAGGCTCCGTTGATGATATTCAAACGCTAGATAACCTCGTGACCGAATAT     780
 I  T  Y  T  G  S  V  D  D  I  Q  T  L  D  N  L  V  T  E  Y     243
AATAAGACCGCGACGATGCCGGTACGGATTCACGTTGATGCTGCCTTTGGTGGCCTGTTC     840
 N  K  T  A  T  M  P  V  R  I  H  V [D] A  A  F  G  G  L  F     263
GCGCCGTTCGTCGATGGCTTTAACCCGTGGGACTTCCGGTTGAAGAACGTGGTTTCCATT     900
 A  P  F  V  D  G  F  N  P  W  D  F  R  L  K  N  V  V  S  I     283
AACGTTTCGGGCCATAAGTACGGGATGGTTTACCCTGGGTTGGGGTGGATAGTTTGGCGG     960
 N  V  S  G [H] [K] Y  G  M  V  Y  P  G  L  G  W  I  V  W  R     303
CACGACACGGCTGATATTTTACCCGCAGAAATGCGATTCCAAGTGCCATATCTAGGTAAG    1020
 H  D  T  A  D  I  L  P  A  E  M  R  F  Q  V  P  Y  L  G  K     323
ACCGTTGATTCAATCGCCATTAACTTCTCACACAGTGGTGCCCATATCAGTGCGCAATAC    1080
 T  V  D  S  I  A  I  N  F  S  H  S  G  A  H  I  S  A  Q  Y     343
TACAATTTCATTCGATTTGGATTATCAGGTTACAAGACGATCATGCAAAATGTTCGGAAG    1140
 Y  N  F  I  R  F  G  L  S  G  Y  K  T  I  M  Q  N  V  R  K     363
GTGTCATTGAAGCTGACGGCAGCTCTGAAAACGTATGGGATTTTCGATATTTTAGTTGAT    1200
 V  S  L  K  L  T  A  A  L  K  T  Y  G  I  F  D  I  L  V  D     383
GGGTCACAGCTACCAATTAACTGTTGGAAACTAGCGGACGATGCGCCGGTTGGTTGGACG    1260
 G  S  Q  L  P  I  N  C  W  K  L  A  D  D  A  P  V  G  W  T     403
TTGTATGATTTGGAGTCCGAGCTGGCTAAGTATGGTTGGCAAGTCCCAGCTTACCCGCTG    1320
 L  Y  D  L  E  S  E  L  A  K  Y  G  W  Q  V  P  A  Y  P  L     423
CCAAAGAATCGCGACGATGTGACAATTAGCCGGATCGTGGTACGCCCATCCATGACCATG    1380
 P  K  N  R  D  D  V  T  I  S  R  I  V  V  R  P  S  M  T  M     442
ACGATTGCCGATGATTTCTTGGATGATTTGAAATTAGCAATTGATGGATTAAATCACACA    1440
 T  I  A  D  D  F  L  D  D  L  K  L  A  I  D  G  L  N  H  T     463
TTTGGCGTGACGACCACCGTTGATCAAGATAACAAGACCACCGTTCGAAGTTAA          1446
 F  G  V  T  T  T  V  D  Q  D  N  K  T  T  V  R  S  *           480
```

Fig. 1. Nucleotide and Deduced Amino Acid Sequences of *gad* B Gene from *Lactobacillus brevis* IFO 12005.

The nucleotide sequence and the deduced amino acid sequence are numbered on the right. The assumed ribosome-binding site is shown by underline. The presumed catalytic amino acid residues (Asp256, His288, and Lys289) are shown by boxes. The accession number of the gene is AB258458.

at a flow rate of 0.2 ml/min. Purified recombinant GAD (120 µg protein in the same buffer) were loaded onto the column, and the apparent molecular weight was estimated from the standard curve.

Results

GAD gene cloning

After screening of a partial DNA library, containing about 4 to 6 kbp *Pst* I/*Sal* I genomic DNA fragments, a 5-kbp DNA fragment containing about 1.6 kbp GAD gene was obtained. The GAD gene of *L. brevis* IFO 12005 was 1,440 bp (ORF), encoding a protein of 480 amino acids (Fig. 1, accession no., AB258458). The nucleotide sequence showed 99% identity with that of *Lactobacillus brevis* ATCC367, but no matching with that of *L. brevis* OPK-3.[26] A deduced amino acid sequence of GAD from *L. brevis* IFO 12005 showed 99.6% identity with that of *L. brevis* ATCC367, 53.1% identity with that of *L. lactis*, and 38.1% identity with that of *E. coli* (Gad B). By multiple alignment analysis reported by Sukhareva *et al.*,[27] highly conserved catalytic amino acid residues were also found in the *L. brevis* GAD. Therefore, Asp 256, His 288, and Lys 289 were assumed to be the catalytic amino acids. They are shown in Fig. 1 (boxed).

Expression and purification of the GAD protein

The expression level of His-tagged GAD protein (GAD-6xHis) in *E. coli* JM109 was very low (Fig. 2A), and low GAD activity was detected. In contrast, the expression level of GAD-6xHis increased drastically

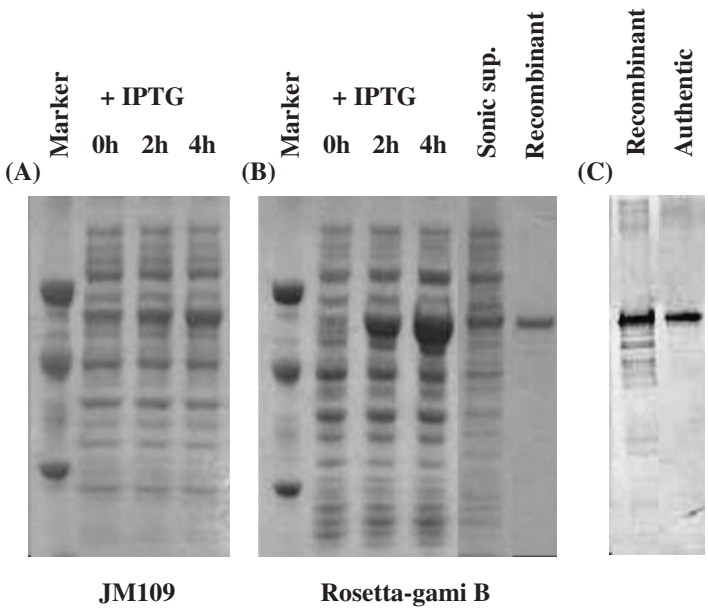

Fig. 2. SDS-Polyacrylamide Gel- and Western Blotting-Analyses of Recombinant GAD Protein.

A 10% polyacrylamide gel was used in analysis. Bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa) were used as protein markers. A, SDS–PAGE analysis of total cell proteins in *E. coli* JM109 after 0, 2, and 4 h, induction with 1 mM IPTG. B, SDS–PAGE analysis of total cell proteins in *E. coli* Rosetta-gami B after 0, 2, and 4 h induction with 1 mM IPTG, sonic supernatant of *E. coli* Rosetta-gami B after overnight induction with 1 mM IPTG, and recombinant GAD purified with a Ni-NTA-agarose column. C, Western blotting analysis of purified recombinant GAD from *E. coli* Rosetta-gami B and purified authentic GAD from *L. brevis* IFO 12005.

when *E. coli* Rosetta-gami B (DE3) pLac I was used as the host strain (Fig. 2B), and higher GAD activity was detected.

A cell-free extract of the cells from 1 liter of culture was applied to the Ni-NTA-agarose column, and about 10 mg of the fusion protein was purified as a single band by Coomassie staining after SDS–PAGE (Fig. 2B). The N-terminal amino acid sequence (five amino acid residues) of the recombinant GAD protein was found to be identical with that of authentic GAD. Western blotting analysis revealed that the purified authentic and recombinant GAD proteins were recognized by anti-GAD antiserum raised against authentic GAD of *L. brevis* IFO12005 (Fig. 2C).

Characteristics of the recombinant GAD

The recombinant GAD, purified as a His-tagged fusion protein, showed optimum pH at 4.5–5.0, and was fully activated after initial treatment with ammonium sulfate (> about 1.8 M) (Fig. 3A, and in Fig. 3B, state 1 to state 2) and the following addition of substrate (Fig. 3B, state 2 to state 3), and the K_m value was 1.4 mM. In contrast, authentic GAD protein showed optimum pH at 4.2–4.6, and was fully activated by similar procedures, with a K_m value of 1.0 mM (Table 1).[28]

The thermal stability of the recombinant GAD was enhanced in the presence of ammonium sulfate at 0.5 M and higher concentrations (Fig. 4).

Gel-filtration column chromatography

The recombinant GAD was eluted from the column as a single peak (Fig. 5A) corresponding to the dimer (apparent molecular weight, 110 kDa) at pH 7.0 (a similar single peak was also detected at pH 5.5),

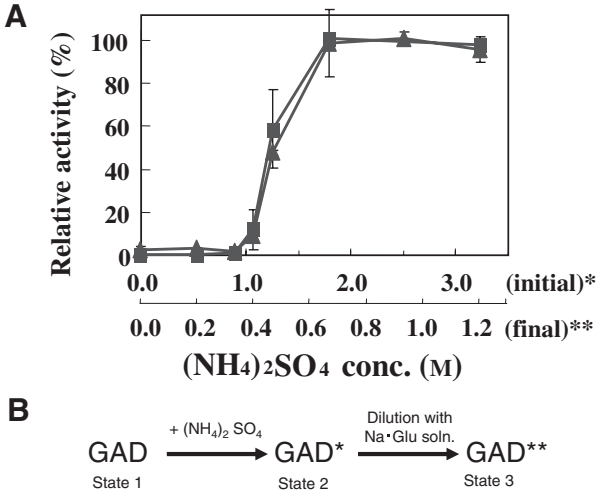

Fig. 3. Effects of Ammonium Sulfate on GAD Activity.

The GAD activity of the authentic GAD (■) and recombinant GAD (▲) was measured at various concentrations of ammonium sulfate. The "initial" scale shows initial concentrations of ammonium sulfate added to the enzyme solution before the addition of the other materials, and the "final" scale shows final concentrations of ammonium sulfate in the reaction mixture after the addition of all materials, such as GAD, PLP, ammonium sulfate, and sodium glutamate. Maximal GAD activity is shown as 100%. Values are averages of three replicates, and standard deviations are indicated by vertical bars.

Table 1. Some Characteristics of Glutamate Decarboxylases from Microorganisms

Origin	Subunit (kDa)	Number of subunit	Opt. pH	Opt. Temp. (°C)	K_m (mM)	*kcat/Km* ($M^{-1}\,s^{-1}$)	References
Lactobacillus brevis (*recombinant*)	54	4	4.2–4.6 (4.5–5.0)	30	1.0 (1.4)	1.4×10^2 (2.6×10^2)	28)
Lactococcus lactis	54	—	4.7	—	0.51	—	15)
Escherichia coli	50	6	4.4	—	1.0	—	21)
Neurospora crassa	33	1	5	—	2.2	—	29)
Aspergillus oryzae	48	6	—	60	13.3	—	19)

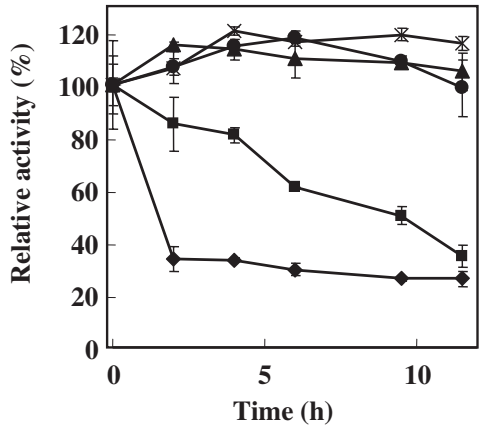

Fig. 4. Effect of Ammonium Sulfate on the Stability of Purified Recombinant GAD.

Purified recombinant GAD was adjusted to various concentrations of ammonium sulfate: ◆, 0 M; ■, 0.25 M; ▲, 0.5 M; ×, 1 M; and ●, 2 M. After treatment at 37 °C at pH 4.6 for the indicated periods, 4 M ammonium sulfate solution was added to each sample in order to adjust the ammonium sulfate concentration to 2.0 M. Then the substrate solution was added, and the remaining activity was measured at pH 4.6 for 1 h. Maximal GAD activity is shown as 100%. Values are the averages of three replicates, and standard deviations are indicated by vertical bars.

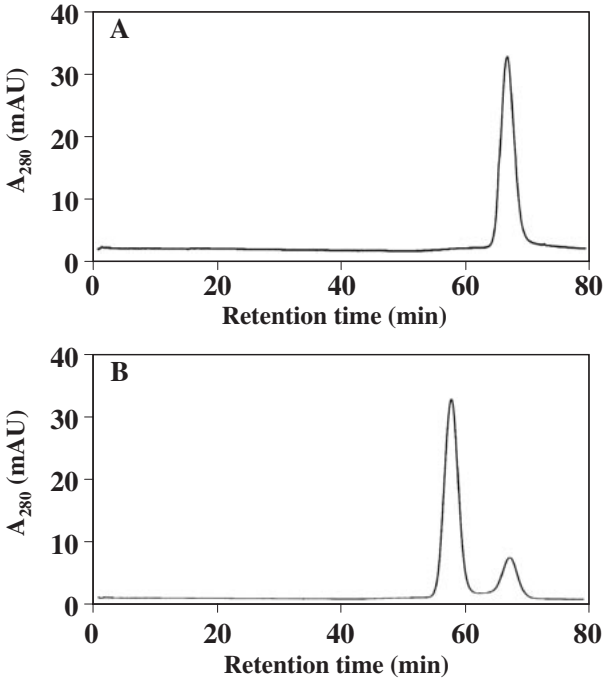

Fig. 5. Gel Filtration Analysis of GADs before (A) and after (B) Treatment with Ammonium Sulfate.

Purified recombinant GAD was analyzed by Superdex 200 10/300 GL column before (A) and after (B) treatment with 2 M ammonium sulfate and subsequent dilution with sodium glutamate-containing buffer to give 0.5 M ammonium sulfate and 1 mM sodium glutamate. For details, see text.

whereas the GAD after ammonium sulfate activation and the following dilution with sodium glutamate was eluted as a tetramer (apparent molecular weight, 250 kDa) at pH 5.5 (Fig. 5B).

CD spectrum analysis

CD spectra of the recombinant GAD were measured in the far UV region (200–250 nm). The CD spectrum of the GAD dimer (Fig. 6c; the sample used was the minor peak in Fig. 5B) and that of the tetramer (active form, in Fig. 6b; the sample used was the major peak in Fig. 5B) at pH 5.5 were similar to each other, but clearly different from that of the GAD dimer at pH 7.0 (the inactive form, in Fig. 6a).

Discussion

We have reported some enzymatic properties of authentic GAD from *L. brevis* IFO12005.[28] The presence of sulfate ions and dimer formation were essential to the enzymatic activity. GADs of microbial origin, such as *E. coli* and *Aspergillus oryzae*, are hexametric except for the GAD of *Neurospora crassa* (Table 1).[15,19,21,28,29] In the case of Gad B from *E. coli*, it has been reported that GAD is stimulated by NaCl.[22] The halide-binding site was identified to be the bases of the N-terminal helices, which are necessary to form two triple-helix bundles of the Gad B hexamer.[22]

In order to characterize the GAD from *L. brevis* IFO12005 in detail, we isolated and cloned its gene. The deduced amino acid sequence showed considerable similarity to Gad B of other microbial species, with *L. brevis* ATCC367 GAD, 99.6%,[30] with *L. lactis* GAD,[14] 53.1% and with *E. coli* Gad B, 38.1%. However, detailed biochemical data have not been reported for the GAD from *L. brevis* ATCC367 or *L. lactis*, except for that from *E. coli*.

In *E. coli* chromosome, two GAD isoforms, *gad* A and *gad* B, are located separately. Another gene, *gad* C,

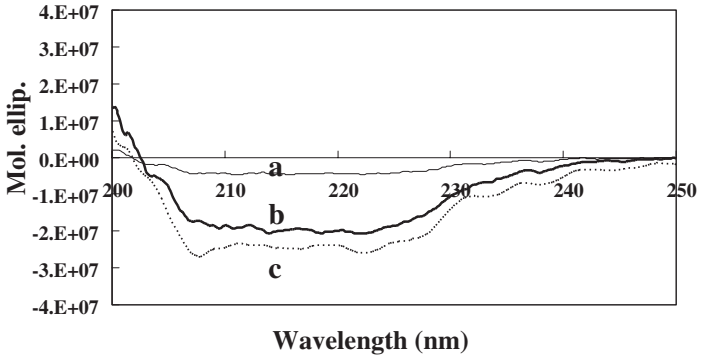

Fig. 6. Circular Dichroism Spectra of Purified Recombinant GAD.

Line a, Purified recombinant GAD (30 µl, 0.56 nmole) and 20 mM phosphate buffer, pH 7.0 (270 µl) were mixed, then the CD spectrum was measured. The final concentration of the enzyme was 100 µg/ml. Line b, The GAD tetramer (0.5 M ammonium sulfate, 0.2 mM PLP, 1 mM sodium glutamate, 50 mM acetate buffer, pH 5.5) fractionated by gel filtration was subjected to measure the CD spectrum. The final concentration of the enzyme was 46 µg/ml. Line c, The GAD dimer (0.5 M ammonium sulfate, 0.2 mM PLP, 1 mM sodium glutamate, 50 mM acetate buffer, pH 5.5) fractionated by gel filtration was subjected to measure the CD spectrum. The final concentration of the enzyme was 10 µg/ml.

encodes a putative glutamate/GABA antiporter. This GAD system of *E. coli* is assumed to control the acidification of cytosol by decarboxylating a glutamate into a neutral GABA through the incorporation of H^+.[23] *L. brevis* IFO12005 had only a single copy gene for *gad* B (data not shown), as did *L. lactis*. A gene similar to *gad C* (accession no., AB258459) was also located very close to the 5′-side of the *gad* B gene (42.5% identity with *L. lactis gad* C). These results suggest that *L. brevis* IFO 12005 has an acid tolerance mechanism similar to *L. lactis*.[15]

The total amount of authentic GAD purified from *L. brevis* IFO12005 was 0.45 mg/l of culture, whereas the recombinant GAD purified from *E. coli* Rosetta gami B harboring the expression plasmid yielded 10 mg/l of culture. Thus we succeeded in getting enough enzyme to study the biochemical properties of GAD.

The K_m value of recombinant GAD (1.4 mM) was slightly higher than that of authentic GAD (1.0 mM). This difference might be due to interference of the C-terminal His-tag of the recombinant GAD of *L. brevis* IFO12005 with the creation of a proper substrate-binding pocket.

Activation of enzyme activity by a high concentration of ammonium sulfate (1.6 M) has been reported for phospholipase D,[31] but the mechanism has not yet been clarified. Initially, we thought that the high concentration of ammonium sulfate might lower the pH of the reaction mixture, and that this low pH might induce conformational changes in *L. brevis* GAD. However, acidic pH treatment (pH 4.0–5.5) of GAD without the addition of ammonium sulfate did not result in activation even after subsequent addition of sodium glutamate. At the second step of activation, dilution of the ammonium sulfate-treated GAD with sodium glutamate solution was essential, because this step was not replaced with distilled water, 50 mM sodium acetate buffer (pH 5.5), or pyridoxal 5′-phosphate solution. Thus it was found that in order to activate *L. brevis* IFO12005 GAD, treatment with a high concentration of ammonium sulfate and subsequent dilution with glutamate solution are essential.

In Fig. 5B, the major peak of the tetramer (retention time, 58 min) showed GAD activity, and in contrast, the minor peak of the dimer (retention time, 68 min) did not show any GAD activity. The dimer was not activated by the addition of ammonium sulfate and subsequent dilution with sodium glutamate. These results suggest that the tetramer is an active form and the dimer is an inactive form. We have reported that dimer formation was essential to activity,[28] but that proved to be a mistake. As far as we know, this is the first report of a tetramer form of GAD from microorganisms (Table 1).[15,19,21,28,31]

CD spectral analyses revealed that the structure of the active form of GAD at pH 5.5 was different from that of the inactive enzyme at pH 7.0. The addition of ammonium sulfate did not cause any significant structural changes at either pH level, suggesting that it did not induce overall structural changes, but did induce subtle structural changes at the active site, probably in the vicinity of the presumed catalytic residues, D256, H288, and K289, of *L. brevis* 12005 GAD. These residues are well conserved among all GADs, even those with low overall similarity.

Consequently we can say that hydrophobic interactions between subunits under a high concentration of ammonium sulfate and proper binding of sodium glutamate to the GAD facilitate making an active tetramer from an inactive dimer. It is very doubtful to expect such a high hydrophobic situation in nature. In order to understand the activation mechanism of the GAD, further study including crystal structural analysis is necessary.

Acknowledgments

We are grateful to Dr. Alexander Wlodawer (National

Cancer Institute, USA) for critical reading, corrections, and helpful comments on a draft of the manuscript.

References

1) Ueno, H., Enzymatic and structural aspects on glutamate decarboxylase. *J. Mol. Cat. B*: *Enzymatic*, **10**, 67–79 (2000).
2) Roberts, E., and Frankel, S., Glutamic acid decarboxylase in brain. *J. Biol. Chem.*, **188**, 789–795 (1951).
3) Stanton, H. C., Mode of action of gamma aminobutyric acid on the cardiovascular system. *Arch. Int. Pharmacodyn. Ther.*, **143**, 195–204 (1963).
4) Lin, Z., Saito, H., Omori, M., Inomata, T., Kato, M., Sawai, Y., Fukatu, S., and Hakamata, K., Effect of gabaron tea on the blood pressure and kidney function of rats loaded with saline. *J. Home Econ. Jpn.* (in Japanese), **51**, 265–271 (2000).
5) Kono, I., and Himeno, K., Changes in γ-aminobutyric acid content during beni-koji making. *Biosci. Biotechnol. Biochem.*, **64**, 617–619 (2000).
6) Saikusa, T., Horino, T., and Mori, Y., Accumulation of gamma-aminobutyric acid (gaba) in the rice germ during water soaking. *Biosci. Biotechnol. Biochem.*, **58**, 2291–2292 (1994).
7) Tramonti, A., Biase, D. De, Giartosio, A., Bossa, F., and John, R. A., The Roles of His-167 and His-275 in the reaction catalyzed by glutamate decarboxylase from *Escherichia coli*. *J. Biol. Chem.*, **273**, 1939–1945 (1998).
8) Biase, D. De, Tramonti, A., John, R. A., and Bossa, F., Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid decarboxylase from *Escherichia coli*. *Protein Expr. Purif.*, **8**, 430–438 (1996).
9) Smith, D. K., Kassam, T., Singh, B., and Elliott, J. F., *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. *J. Bacteriol.*, **174**, 5820–5826 (1992).
10) Maras, B., Sweeney, G., Barra, D., Bossa, F., and John, R. A., The amino acid sequence of glutamate decarboxylase from *Escherichia coli*: evolutionary relationship between mammalian and bacterial enzymes. *Eur. J. Biochem.*, **204**, 93–98 (1992).
11) Sanders, J. W., Leenhouts, K., Burghoorn, J., Brands, J. R., Venema, G., and Kok, J., A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. *Mol. Microbiol.*, **27**, 299–310 (1998).
12) Sanders, J. W., Venema, G., Kok, J., and Leenhouts, K., Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene. *Mol. Gen. Genet.*, **257**, 681–685 (1998).
13) Sanders, J. W., Venema, G., and Kok, J., A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*. *Appl. Environ. Microbiol.*, **63**, 4877–4882 (1997).
14) Nomura, M., Kobayashi, M., Ohmomo, S., and Okamoto, T., Inactivation of the glutamate decarboxylase gene *Lactococcus lactis* subsp. *cremoris*. *Appl. Environ. Microbiol.*, **66**, 2235–2237 (2000).
15) Nomura, M., Nakajima, I., Fujita, Y., Kobayashi, M., Kimoto, H., Suzuki, I., and Aso, H., *Lactococcus lactis* contains only one glutamate decarboxylase gene. *Microbiol.*, **145**, 1375–1380 (1999).
16) Hao, R., and Schmit, J. C., Cloning of the gene for glutamate decarboxylase and its expression during conidiation in *Neurospora crassa*. *Biochem. J.*, **293**, 735–738 (1993).
17) Cotter, P. D., Gahan, C. G. M., and Hill, C., A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol. Microbiol.*, **40**, 465–475 (2001).
18) Waterman, S. R., and Small, P. L. C., Identification of the promoter regions and σ^s-dependent regulation of the *gadA* and *gadBC* genes associated with glutamate-dependent acid resistance in *Shigella flexneri*. *FEMS Microbiol. Lett.*, **8**, 155–160 (2003).
19) Tsuchiya, K., Nishimura, K., and Iwahara, M., Purification and characterization of glutamate decarboxylase from *Aspergillus oryzae*. *Food Sci. Technol. Res.*, **9**, 283–287 (2003).
20) Kato, Y., Kato, Y., Furukawa, K., and Hara, S., Cloning and nucleotide sequence of the glutamate decarboxylase-encoding gene *gadA* from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.*, **66**, 2600–2605 (2002).
21) Fonda, M. L., L-Glutamate decarboxylase from bacteria. *Meth. Enzymol.*, **113**, 11–16 (1985).
22) Gut, H., Pennacchietti, E., John, R. A., Bossa, F., Capitani, G., Biase, D. De, and Grutter, M. G., *Escherichia coli* acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB. *EMBO J.*, **25**, 2643–2651 (2006).
23) Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L., and Slonczewski, J. L., A glutamate-dependent acid resistance gene in *Escherichia coli*. *J. Bacteriol.*, **178**, 3978–3981 (1996).
24) Dutyshev, D. I., Darii, E. L., Fomenkova, N. P., Pechik, I. V., Polyakov, K. M., Nikonov, S. V., Andreeva, N. S., and Sukhareva, B. S., Structure of *Escherichia coli* glutamate decarboxylase (GADα) in complex with glutarate at 2.05 Å resolution. *Acta Cryst.*, **D61**, 230–235 (2005).
25) Hayakawa, K., Ueno, Y., Kawamura, S., Taniguchi, R., and Oda, K., Production of γ-aminobutyric acid by lactic acid bacteria. *Seibutsu-kogakukaishi* (in Japanese), **75**, 239–244 (1997).
26) Park, K. B., and Oh, S. H., Cloning, sequencing and expression of a novel glutamate decarboxylase gene from a newly isolated lactic acid bacterium, *Lactobacillus brevis* OPK-3. *Bioresour. Technol.*, **98**, 312–319 (2007).
27) Sukhareva, B. S., and Mamaeva, O. K., Glutamate decarboxylase: computer studies of enzyme evolution. *Biochemistry* (Moscow), **67**, 1428–1436 (2002).
28) Ueno, Y., Hayakawa, K., Takahashi, S., and Oda, K., Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO12005. *Biosci. Biotechnol. Biochem.*, **61**, 1168–1171 (1997).
29) Hao, R., and Schmit, J. C., Purification and characterization of glutamate decarboxylase from *Neurospora crassa* conidia. *J. Biol. Chem.*, **266**, 5135–5139 (1991).
30) Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin,

K., Lee, J.-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., and Mills, D., Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA*, **103**, 15611–15616 (2006).

31) Nakamura, S., Shimooku, K., Akisue, T., Jinnai, H., Hitomi, T., Kiyohara, Y., Ogino, C., Yoshida, K., and Nishizuka, Y., Mammalian phospholipase D: activation by ammonium sulfate and nucleotides. *Proc. Natl. Acad. Sci. USA*, **92**, 12319–12322 (1995).