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Characterization of Glutamate Decarboxylase from a High γ-Aminobutyric Acid (GABA)-Producer, *Lactobacillus paracasei*

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γ-Aminobutyric acid (GABA) has several physiological functions in humans. We have reported that *Lactobacillus paracasei* NFRI 7415 produces high levels of GABA. To gain insight into the higher GABA-producing ability of this strain, we analyzed glutamate decarboxylase (GAD), which catalyzes the decarboxylation of L-glutamate to GABA. The molecular weight of the purified GAD was estimated to be 57 kDa by SDS–PAGE and 110 kDa by gel filtration, suggesting that GAD forms the dimer under native conditions. GAD activity was optimal at pH 5.0 at 50 °C. The Km value for the catalysis of glutamate was 5.0 mM, and the maximum rate of catalysis was 7.5 μmol min^{-1} mg^{-1}. The N-terminal amino acid sequence of GAD was determined, and the gene encoding GAD from genomic DNA was cloned. The findings suggest that the ability of *Lb. paracasei* to produce high levels of GABA results from two characteristics of GAD, *viz.*, a low Km value and activity at low pH.

Key words: glutamate decarboxylase (GAD); *Lactobacillus paracasei*; γ-aminobutyric acid (GABA)

γ-Aminobutyric acid (GABA) is a nonprotein amino acid widely distributed in nature.[1)] It has several physiological functions, such as neurotransmission and the induction of hypotensive, diuretic, and tranquilizer effects.[2,3)] Due to the physiological functions of GABA, the development of functional foods containing GABA in high concentrations has been actively pursued.[4,5)] GABA production by various microorganisms has been reported, including bacteria,[6,7)] fungi,[8)] and yeasts.[9)]

We focused on the GABA production ability of lactic acid bacteria (LAB) because LAB possess commercial potential as a starter of production in fermented foods such as pickled vegetables and fermented meats and fishes. We have reported that *Lactobacillus paracasei* isolated from a traditional Japanese fermented fish (*funa-sushi*) showed high GABA-producing ability.[10)] The production of GABA in *Lb. paracasei* was superior to that in *Lb. brevis*, a known GABA producer, but GABA production by *Lb. paracasei* has not yet been characterized. We consider *Lb. paracasei* to be a good model for investigating the high levels of GABA production in LAB.

Glutamate decarboxylase (GAD; EC 4.1.1.15) is the enzyme that catalyzes the decarboxylation of L-glutamate to GABA. It has been reported that GAD is present in the mammalian brain,[11,12)] plants,[13,14)] *Escherichia coli*,[15)] *Aspergillus*,[16)] and LAB.[17)] The purification and enzymatic properties of GAD in such LAB species as *Lactobacillus* and *Lactococcus* have been reported. Ueno *et al.*[18)] purified GAD from *Lb. brevis* and then determined its biochemical characteristics. Park and Oh[19)] reported the cloning and sequencing of the GAD gene from a newly isolated *Lb. brevis*. Nomura *et al.*[20)] characterized GAD and cloned the gene responsible for GAD activity in *L. lactis*, and found that *L. lactis* contained one gene encoding GAD.

In this report, we describe a four-step procedure for the purification of *Lb. paracasei* GAD to homogeneity, and we discuss our investigation of its biochemical characteristics. The biochemical characteristics of *Lb. paracasei* GAD were compared with those of GAD from other LAB species. We also explain the cloning and sequence of the gene encoding GAD.

Materials and Methods

Strains and media. Lb. paracasei NFRI7415 was used in enzyme production and gene cloning. *Escherichia coli* DH5α was used in plasmid manipulations.

Lactobacilli MRS medium (Difco Laboratories, Detroit, MI) was used in the cultivation of *Lb. paracasei*. LB medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) was used in the cultivation of *E. coli*.

Purification of GAD. A pre-culture of *Lb. paracasei* was grown to the stationary phase at 37 °C for 20 h in

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MRS medium. Two ml of the pre-culture was inoculated into 1 liter of MRS medium containing 100 mM of glutamate, and the inoculate was incubated at 37 °C for 72 h. Our previous study showed that GABA production occurred in MRS medium after a cultivation of period of at least 72 h.[10] After cultivation, the cells were collected by centrifugation (8,000 × *g* for 10 min at 4 °C) and washed twice with phosphate-buffered saline (PBS; pH 7.0) containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter. The cells were suspended in 50 ml of PB (phosphate buffer (PB): 20 mM sodium phosphate pH 7.0), containing 0.1 mM pyridoxal 5′-phosphate (PLP) and 0.1 mM 2-mercaptoethanol. The suspension was incubated with 0.2 mg/ml of lysozyme at 37 °C for 15 min. After incubation, 10 g of glass beads (100 μm in diameter, Merck, Darmstadt, Germany) were added to the cell suspension, and the cells were disrupted by shaking for 30 s (10 times) using a homogenizer (type MSK, Braun, Frankfurt, Germany) at 4 °C. Crude extracts were obtained by centrifugation at 10,000 × *g* for 10 min at 4 °C.

Ammonium sulfate was added to the crude extract to give a final concentration that was 80% saturated. The precipitate was collected by centrifugation (10,000 × *g* for 10 min at 4 °C) and dissolved in 5 ml of PB. The enzyme solution was dialyzed against PB at 4 °C overnight. The dialysate sample was adjusted to 0.4 M ammonium sulfate by adding PB containing 0.8 M ammonium sulfate. The sample was applied to a column (4 × 80 cm) of Butyl-Toyopearl 650M (Tosoh, Tokyo) equilibrated with PB containing 0.8 M ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate (0.8 to 0 M). The fractions containing GAD activity (18 ml) were pooled, and ultrafiltration was performed at 4 °C for the concentration of proteins. Then the samples were loaded onto DEAE-Sepharose FF columns (GE Healthcare Bio-Science, Piscataway, NJ) equilibrated with PB. Each column was washed with PB, and the enzyme was eluted with a linear gradient of NaCl (0 to 1 M). The fractions containing GAD activity (16 ml) were pooled and dialyzed against PB. The dialysate was loaded onto Mono Q 5/50 GL columns (GE Healthcare Bio-Science) equilibrated with PB, and the samples were eluted with a linear gradient of NaCl (0.4 to 0.6 M). The fraction containing GAD activity (2 ml) was used in further analyses.

Enzyme and protein assays. An enzyme solution was incubated with 50 mM of glutamate and 10 μM of PLP in 0.2 M pyridine-HCl buffer (pH 5.0) at 37 °C for 10 min. The enzyme reactions were stopped by boiling the samples for 5 min. The reaction mixture was then analyzed by thin-layer chromatography (TLC) or with an amino acid analyzer (LC-11A, Yanagimoto, Kyoto, Japan). To determine the activity in the fractions eluted by column chromatography, TLC using Silica gel 60 F245 (Merck) was carried out with the following solvent system: CH_3OH: $CHCl_3$ (9.5:0.5, v/v). An amino acid analyzer was used specifically to measure GAD activity as the amount of GABA produced. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA in 1 min. The protein concentration was determined with BCA protein assay reagent (Pierce, Rockford, IL).

SDS–PAGE of purified GAD. SDS–PAGE of fractions eluted by column chromatography was performed by the method of Laemmli[21] using 10% polyacrylamide gel. The standard marker of molecular weight was obtained from Bio-Rad Laboratories (Hercules, CA). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 (CBB) and destained with 30% methanol.

N-Terminal amino acid sequence. The purified GAD was separated on 10% SDS–PAGE gel and electrotransferred to a PVDF membrane. The membrane was stained with 0.1% CBB, destained with 60% methanol, and washed in distilled water. The band of GAD was sliced from the membrane, and its N-terminal sequence was determined using a protein sequencer (HP G1005A protein sequencing system, Hewlett Packard, Palo Alto, CA).

Analytical gel filtration. The purified GAD (0.2 ml) was loaded on a column of Sephacryl S-100 HR 16/60 equilibrated with PB containing 0.15 M NaCl and EDTA, and eluted with the same buffer at a flow rate of 0.5 ml/min. β-Amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were used as molecular weight standards. All standards were purchased from Sigma-Aldrich (St. Louis, MO).

Effect of temperature and pH on GAD activity. The optimum temperature of GAD activity was determined during a 30 min exposure to a temperature range of 20 °C to 60 °C in 0.2 M pyridine-HCl buffer (pH 5.0) with 50 mM of glutamate and 10 μM of PLP. The thermal stability of the enzyme was determined by incubating the enzyme in a temperature range of 20 °C to 60 °C for 1 h at pH 5.0, and the residual activity of the enzyme was measured at 37 °C.

The optimum pH of pH stability of GAD activity was determined within a range of pH 3.5 to 6.0 in 0.2 M pyridine-HCl buffer supplemented with 50 mM of glutamate and 10 μM PLP at 37 °C. The pH stability of the enzyme was determined by incubating it at each pH with 0.2 M pyridine-HCl buffer for 1 h at 4 °C, and its residual activity was measured.

Cloning of the gene encoding GAD. Genomic DNA was isolated from *Lb. paracasei* NFRI 7415 using an InstaGene kit (Bio-Rad) and was then used as the template DNA in PCR cloning. To amplify the internal

fragments of the gene encoding GAD, primers designed from highly conserved regions of GAD were used. The nucleotide sequences of these degenerate primers were as follows: forward (F3) 5′-CAR(A/G)GTN(A/C/G/T)-TGY(C/T)TGGGAR(A/G)AA-3′ and reverse (R6) 5′-GGR(A/G)TAN(A/C/G/T)ACN(A/C/G/T)AR(A/G)N-(A/C/G/T)CCR(A/G)TAY(C/T)TTR(A/G)TG-3′. PCR was initiated with 4 min of denaturation at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 40 °C, and 1 min at 72 °C using a Gene Amp PCR System (Applied Biosystems, Lincoln, CA). The PCR product was purified using a gel extraction kit (Qiagen, Hilden, Germany). The purified product was ligated into the pTA2 vector (Toyobo, Osaka, Japan) using T4 ligase (Toyobo), and the ligated vector was then introduced into *E. coli* DH5α. The cloned DNA was sequenced as described by Sanger *et al.*[22] using an ABI PRIZM 310 genetic analyzer with a BigDye Terminator kit (Applied Biosystems).

To isolate the full-length GAD gene, inverse PCR was carried out.[23] Restriction enzymes suitable for digestion of genomic DNA were selected by Southern blot analysis. Self-ligation was carried out using the genomic DNA digested with *Pst*I.

Primers for the inverse PCR primers were designed based on the nucleotide sequence of the cloned internal fragment and the N-terminal amino acid sequence determined by protein sequencing. The sequences of the primers were as follows: gF3, 5′-GACTTGGAA-GGTGAACTTGC-3′; gN1-2, 5′-GCAATACTAGCTG-GCATTGG-3′; gF6, 5′-GGAGGATCTAAAGATGGC-CA-3′; and gN1-1, 5′-GGCAACTCTTCAGTTGGCAA-3′. The first PCR was carried out using gF3 and gN1-2 primers and self-ligated genomic DNA as the template. The PCR cycling conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. The products of the first PCR were used as a template for the second PCR. The second PCR was carried out using gF6 and gN1-1 primers. The second PCR product was purified and ligated into pGEM-T Easy vector (Promega, Madison WI). The DNA sequence of cloned fragments was determined, and the sequence was analyzed with Genetyx software (Software Development, Tokyo) or Clustal W software.

Results

Purification of GAD from Lb. paracasei

Purification of GAD in *Lb. paracasei* was attempted using the following approaches: ammonium sulfate precipitation, hydrophobic chromatography, and anion-exchange chromatography (*viz.*, DEAE-Sepharose and Mono-Q chromatography). GAD was eluted by Mono-Q chromatography from the fraction in which the NaCl concentration was 0.44 M. The purity of fractions containing GAD activity was confirmed by SDS–PAGE. The SDS–PAGE analysis of the fractions detected a single band, the molecular weight of which was approximately 57 kDa, in fraction 16; this result indicated that GAD was purified to homogeneity (Fig. 1). The purification of GAD in *Lb. paracasei* is summarized in Table 1. The data revealed that the specific activity of GAD increased approximately 27-fold, with a yield of 6.2%. We used the eluted active fraction (fraction 16) from Mono-Q column chromatography as the purified enzyme preparation for the subsequent studies.

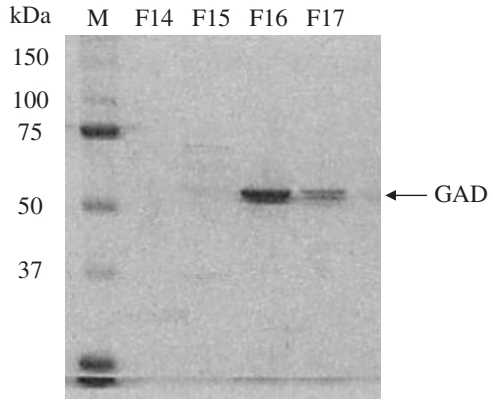

Fig. 1. SDS–PAGE of Factions (F14-F17) Exhibiting GAD Activity Eluted with Mono-Q Column Chromatography.

The fractions (F14-F17) eluted by Mono-Q column chromatography were subjected to SDS–PAGE. M, molecular mass standard.

The molecular weight of native GAD was determined by gel filtration analysis. A single peak with GAD activity was detected at a molecular weight of approximately 110,000 (data not shown). These results, taken together with those of the SDS–PAGE analysis, reveal that the *Lb. paracasei* GAD forms a dimer under native conditions.

Properties of GAD in Lb. paracasei

The pH and temperature dependencies of GAD activity were examined, and the results are shown in Fig. 2. The findings revealed optimum activity at pH 5.0 (Fig. 2A), and the optimum temperature for GAD activity was observed at 50 °C with 30-min reactions (Fig. 2B). The optimum pH for GAD activity in *Lb. paracasei* differed from that previously reported for GAD from other LAB.[18,20] The enzyme was active within a broad pH range as compared with GADs from other LAB species.

The pH and thermal stability of GAD activity are shown in Fig. 2C and D, respectively. The enzyme was stable within a pH range of 4.5 to 5.0, but was unstable below pH 4.0 and above pH 5.5. It was found to be stable within a temperature range of 20 to 40 °C, as shown in Fig. 2D.

The influence of salts on the activity of *Lb. paracasei* GAD was also investigated. We found that 10 mM of ammonium ions and 10 mM of calcium ions enhanced GAD activity by 132% and 114% respectively, as compared with the activity in the no-addition control. However, 10 mM of sodium chloride inhibited GAD

Table 1. Purification of GAD from *Lb. paracasei*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	199	124	0.62	100	1.0
Ammonium sulfate	33	52	1.55	41.9	2.5
Butyl-Toyopearl	8.6	33	3.86	26.8	6.2
DEAE-Sepharose	1.0	11.4	11.6	9.2	18.7
Mono-Q	0.2	3.2	16.7	6.2	26.9

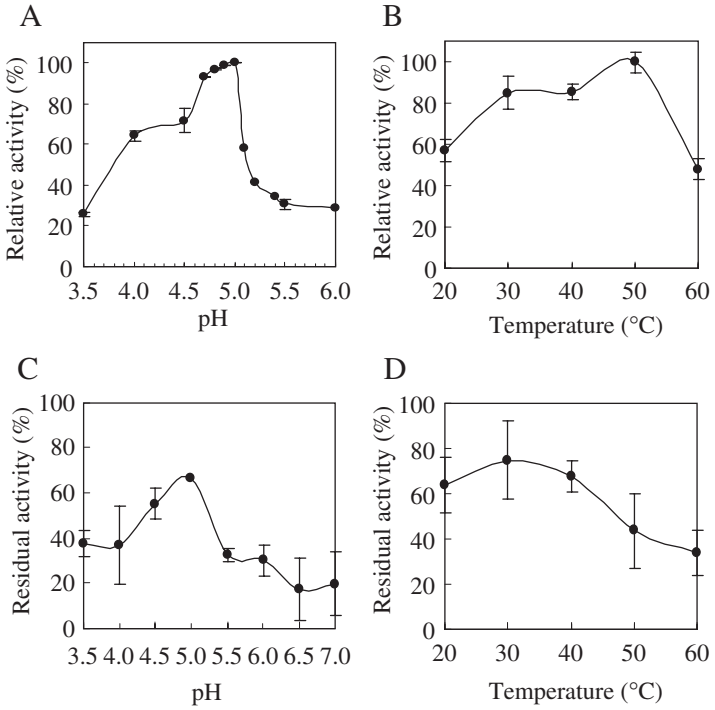

Fig. 2. Effects of pH and Temperature on GAD Activity.

A, Optimum pH. Enzymatic activity was measured in 0.2 M pyridine-HCl buffer at various pHs. B, Optimum temperature. The activity of the enzyme was measured in 0.2 M pyridine-HCl buffer for 30 min, pH 5.0, at various temperatures. C, Effects of pH. The activity of the enzyme was measured in 0.2 M pyridine-HCl buffer at 37 °C after preincubation of GAD at different pHs at 4 °C for 1 h. D, Effects of temperature. The activity of the enzyme was measured in 0.2 M pyridine-HCl buffer at 37 °C after preincubation of GAD at different temperatures and at pH 5.0 for 1 h. Relative GAD activities are shown, with the maximum level of activity represented as 100%. Data from three independent experiments are expressed as mean ± SD.

activity, and the residual activity was 38.5%. GAD activity was also inhibited by 1 mM and 10 mM of EDTA; residual activities of 66.8% and 44.5% respectively were observed. These results suggest that other ions were involved in GAD activity. The *K*m and *V*max values were determined for the reaction using glutamate as a substrate, and the values were calculated from a Lineweaver-Burk plot. The *K*m and *V*max of the enzyme were calculated to be 5.0 mM and 7.5 μmol min^{-1} mg^{-1} respectively.

Sequencing of N-terminal amino acids

The N-terminal sequence of GAD produced by *Lb. paracasei* was determined with a protein sequencer. It appeared that the N-terminal sequence was SEKNDEQMI. Protein homology searches using BLAST were carried out, but no sequences that showed higher homology with the N-terminal sequence of GAD were found.

Cloning and sequencing of the gene encoding GAD

Cloning of the gene encoding the GAD of *Lb. paracasei* was attempted. First an internal fragment of the gene was obtained by PCR using degenerate primers designed based on the conserved sequences in GAD from *Lb. brevis*, *L. lactis*, and *Lb. plantarum*. A 360-bp fragment was obtained and sequenced (data not shown). The amino acid sequence deduced from the DNA sequence of the internal fragment showed high similarity with the sequence GAD of *L. lactis* and *Lb. plantarum*. To isolate the full-length gene encoding GAD, inverse PCR was carried out using primers designed based on the determined N-terminal amino acid sequence and the nucleotide sequences of the internal fragments. The PCR

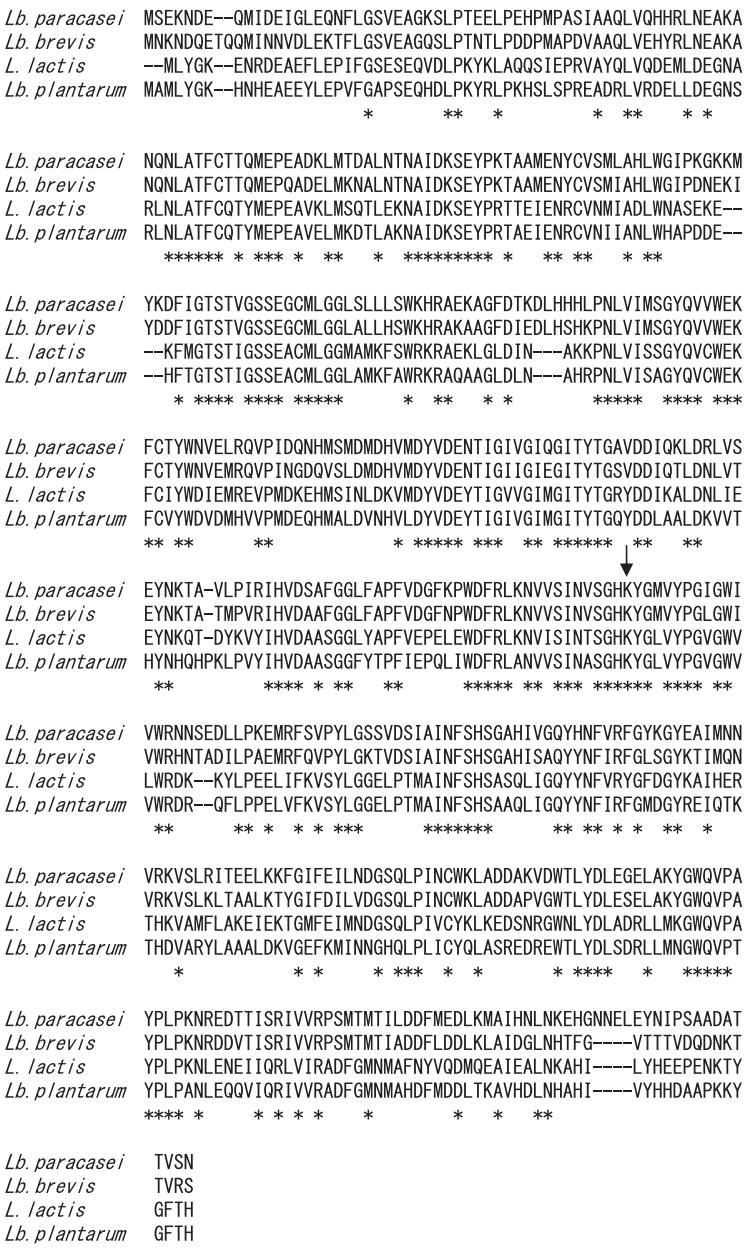

Fig. 3. Comparison of the Deduced Amino Acid Sequence of GadB of *Lb. paracasei* with the GAD Sequences of Other Lactic Acid Bacteria.
The deduced amino acid sequence was analyzed using Clustal W (1.83). Residues conserved in GAD from LABs are marked by asterisks. The arrow indicates the active site for PLP binding.

product containing the full coding region of the gene was obtained. The nucleotide sequencing suggested that this PCR product contained the full length of the gene encoding GAD (designated *gadB*). The results indicate that the *gadB* gene consists of 1,443 bases, which encode a protein of 481 amino acid residues. The predicted molecular mass of the protein was 54.3 kDa. The N-terminal sequence deduced from the sequence of *gadB* and the sequence determined by protein sequencing were identical. Comparison of the amino acid sequence deduced from *gadB* with the protein sequences of other forms of GadB from other LAB demonstrated that the *gadB* product shared a high level of homology with GadB from other LAB (Fig. 3). A computer-based homology search using BLAST revealed that the sequence of GadB shared 79% and 52% homology with the GAD of *Lb. brevis* ATCC 367 and the GAD of *L. lactis* subsp. *lactis* 01-7 respectively. The product of *gadB* of *Lb. paracasei* has the consensus lysyl residue known to be the active site of PLP binding (Fig. 3). The sequence of the *gadB* gene of *Lb. paracasei* was deposited in the DDBJ database under accession no. AB295641.

Discussion

We have reported that *Lb. paracasei* NFRI 7415 produces relatively high levels of GABA.[10] In this

Table 2. Properties of GAD from *Lb. paracasei*, *Lb. brevis*, and *L. lactis*

	Lb. paracasei[a]	*Lb. brevis*[b]	*L. lactis*[c]
Molecular weight (kDa)			
SDS–PAGE	57	60	54
Gel filtration	110	120	ND
Optimum pH	5.0	4.2	4.7
Optimum temperature	50 °C	30 °C	ND
*K*m value (mM)	5.0	9.3	0.51
Activation by 10 mM NaCl	−	−	ND
Activation by 10 mM $(NH_4)_2SO_4$	+	+	ND
Activation by 10 mM $CaCl_2$	+	ND	ND
Production of GABA (μg/ml)	6180 [100][10)]	5090 [100][10)]	69.6 [0][25)]
[glutamate (mM)[d]]	950 [10][10)]		54.6 [20][29)]

ND, not determined.
[a]Data obtained in this study.
[b]Data from Ueno *et al.*[18)]
[c]Data from Nomura *et al.*[20)]
[d]Glutamate concentrations in the growth medium.

study, we examined the biochemical characteristics of glutamate decarboxylase (GAD) in *Lb. paracasei*. Table 2 summarizes the biochemical characteristics of GAD from *Lb. paracasei* and describes its differences from *Lb. brevis* (a high GABA-producing strain) and *L. lactis* (a low GABA-producing strain) GAD. The molecular weight of GAD was estimated to be 57 kDa by SDS–PAGE and 110 kDa by gel filtration. These data strongly suggest that the GAD of *Lb. paracasei* forms a dimer under native conditions. GAD dimer formation has also been reported in *Lb. brevis*.[18)] In *Lactobacillus* species, the dimer formation of GAD might be conserved, whereas the GAD of *E. coli* forms a hexamer.[24)]

The optimum temperature for the GAD activity of *Lb. paracasei* was 50 °C, much higher than that of *Lb. brevis*. This optimum temperature is potentially of advantage in commercial applications of this enzyme, because the higher temperature prevents the growth of contaminants. The enzyme thus might be useful in GABA production using a bio-reactor or batch reaction. However, because the enzyme was not stable at 50 °C, further improvement by modification or mutation may be necessary to achieve greater stabilization at 50 °C.

The optimal pH of GAD in *Lb. paracasei* was pH 5.0, somewhat higher than that of either *Lb. brevis* or *L. lactis*, but the pH range for the maintenance of activity was relatively broad. The GAD activity showed a bell-shaped pH profile from pH 4.5 to 5.5, but it lacked pH dependence between 4.0 and 4.5. Because GAD activity at pH 4.0 is relatively high, GAD does not exhibit a typical bell-shaped pH profile. In high GABA-producing strains (*Lb. paracasei* and *Lb. brevis*), GAD activity was still observed at pH 4.0,[18)] but very low levels of GAD activity have been observed at pH 4.0 in a low GABA-producing strain (*L. lactis*).[25)] These results suggest that low-pH GAD activity might be important for producing high levels of GABA in LAB. In LAB and *E. coli*, GABA production is among the most important mechanisms for achieving acid resistance.[26–28)] Since *Lb. paracasei* increases acidity *via* growth, it has been suggested that it produces GABA in order to increase the pH of the growth medium.[10)]

The *K*m value of GAD from *Lb. paracasei* was much lower than that from *Lb. brevis*. This characteristic might account for the effective conversion from glutamate to GABA, but the *K*m value of GAD from *L. lactis* was lower than that from *Lb. paracasei*. In *L. lactis*, the pH range for GAD activity was relatively narrow. Hence high GAD activity at optimum pH might be required for GABA synthesis in *L. lactis*. To produce high amounts of GABA, both low pH activity and a low *K*m value might be necessary GAD characteristics.

We cloned the gene encoding GAD (*gadB*) of *Lb. paracasei*, and found that the predicted molecular mass (54.3 kDa) was similar to that of the dimerized native form (110 kDa). The deduced amino acid sequence was compared with those of GAD from other LAB. Although these genes shared a high level of homology, the N-terminal and C-terminal regions were not conserved (Fig. 3). It is possible that differences in primary structure might contribute to the high GABA-producing ability of some species. We searched the conserved sequence upstream of *gadB* and found a ribosome binding sequence (GGAGG), but we did not find any possible promoter sequences. We speculated that *gadB* and the other genes located upstream of it might have an operon structure. It has been reported that *gadB* and *gadC* (encoding the antiporter that exchanges glutamate for GABA) form an operon structure in *L. lactis*.[20)] At present, it is unknown whether *gadC* exists upstream of *gadB* in *Lb. paracasei*.

In this study, we identified the biochemical characteristics and putative primary structure of GAD. Various forms of GAD from high GABA-producing strains (*Lb. paracasei* and *Lb. brevis*) were found to have similar characteristics, but there were some notable differences in terms of optimum pH and temperature. Possible reasons for the high levels of GABA production

by *Lb. paracasei* include GAD properties such as low pH activity and high affinity to glutamate. Expression of *gadB* might be important for achieving relatively high GABA production. To gain further insight into the high GABA productivity of this strain, a gene expression analysis of *gadB* and analysis of the transcriptional and translational regulation of GAD are currently underway.

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