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Purification and Characterization of Glutamate Decarboxylase from *Lactobacillus brevis* IFO 12005

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Glutamate decarboxylase (GAD) [EC 4.1.1.15] was purified from a cell-free extract of *Lactobacillus brevis* IFO 12005 by chromatographies on Sephadex G-100, DEAE-Sepharose CL-6B, and Mono Q. About 9 mg of purified GAD was obtained from 90.2 g of wet cells. The purified preparation showed a single protein band on SDS–PAGE. The molecular weights of purified GAD by SDS–PAGE and gel filtration on Superdex 200 were 60,000 and 120,000, respectively, indicating that GAD from *L. brevis* exists as a dimer. The N-terminal amino acid sequence of the purified GAD was NH_2-Met-Asn-Lys-Asn-Asp-Gln-Glu-Gln-Thr-. The optimum pH and temperature of GAD were at pH 4.2 and at 30°C. The GAD activity was increased by the addition of sulfate ions in a dose-dependent manner. The order of effect was as follows: ammonium sulfate > sodium sulfate > magnesium sulfate, indicating that the increase of hydrophobic interaction between subunits causes the increase of GAD activity. The purified GAD reacted only with L-glutamic acid as a substrate and the K_m, k_{cat}, and k_{cat}/K_m values were 9.3 mM, 6.5 s^{-1}, and 7×10^2 M^{-1} s^{-1}, respectively.

Key words: glutamate decarboxylase; gamma amino-*n*-butyric acid; *Lactobacillus brevis*; purification; characterization

Glutamate decarboxylase (GAD) is a pyridoxal enzyme and produces gamma amino-*n*-butyric acid (GABA) from glutamate. GAD and GABA are distributed widely in the biological world from microorganisms to mammals. GABA has several physiological functions such as neurotransmitting,[1] hypotensive, and diuretic effects.[2] Among the physiological functions of GABA, the hypotensive effects of GABA in foods have been reported. Gabaron tea[3] and red mold rice[4,5] contained high amounts of GABA and have anti-hypertensive effects for humans. Moreover, it has been reported that GABA accumulates in rice germ during water soaking.[6]

The enzyme properties and molecular cloning of GAD genes in *Escherichia coli*,[7–11] and *Neurospora crassa*,[12,13] have been reported. Recently, we have reported the existence of GAD in lactic acid bacteria isolated from such foods as Kimchi.[14] Moreover, we have found that *Lactobacillus brevis* IFO 3345, IFO 3960, IFO 12520, and IFO 12005 produced GABA.[14] These results indicate that these microorganisms contained high amounts of GAD. However the enzyme properties of GAD from *L. brevis* have not been studied.

In this study, we have purified GAD from *L. brevis* IFO 12005 and discovered some of its properties.

Materials and Methods

Materials. Sephadex G-100, DEAE-Sepharose CL-6B, and Mono Q HR 5/5 were obtained from Pharmacia Biotech. Bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were from Sigma. Other reagents were obtained from Nacalai Tesque.

Cultivation. A preculture of *L. brevis* IFO 12005 was grown stationarily at 30°C for 24 h in GYP medium (1% glucose, 1% yeast extract, 0.5% polypeptone, 0.2% sodium acetate, 20 ppm $MgSO_4 \cdot 7H_2O$, 1 ppm $MnSO_4 \cdot 4H_2O$, 1 ppm $FeSO_4 \cdot 7H_2O$, 1 ppm NaCl, pH 6.8). The culture was inoculated into the same medium containing 1% sodium glutamate in a jar fermenter (30 liters) containing 20 liters of culture medium and cultivated at 30°C for 48 h without aeration or agitation. After the cultivation, cells were collected by centrifugation (8000 × *g*, 30 min).

Enzyme assay. Enzyme solution (0.1 ml) was mixed with 0.1 ml of 4 M ammonium sulfate and 1.3 ml of substrate solution (0.2 M pyridine–HCl, pH 4.6, containing 0.4 M NaCl and 20 mM sodium glutamate) and incubated at 37°C for 60 min. After the incubation, the reaction was stopped in boiling water for 5 min. Then the produced GAD was analyzed by TLC and measured by HPLC. TLC was done using Kiesel gel F254 (Merck). The solvent system was used *n*-BuOH–AcOH–H_2O (3 : 2 : 1, v/v). The amino acid analysis was done with a Shimadzu HPLC LC-9A. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA per min.

SDS–PAGE of the purified GAD. SDS–PAGE of the purified GAD was done by the method of Laemmli[15] using 10% polyacrylamide gel. Bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrolase (31 kDa), soybean trypsin inhibitor (20 kDa), and lysozyme (14 kDa) were used as molecular mass standards. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

Analytical gel filtration. The purified GAD (0.2 ml) was put on a column of Superdex 200 HR10/30 equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM pyridoxal-5′-phosphate and 0.1 mM 2-mercaptoethanol (Buffer A) containing 0.15 M NaCl and 5 mM EDTA, and eluted at a flow rate of 0.5 ml per min. Rabbit IgG (160 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and aprotinin (6.5 kDa) were used as molecular mass standards.

N-Terminal amino acid sequence. The purified GAD was electrophoresed

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Abbreviations: GAD, glutamate decarboxylase; GABA, gamma amino-*n*-butyric acid.

on SDS-polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane. The protein on the membrane was stained with Coomassie brilliant blue R-250. The protein band was sequenced directly with an Perkin Elmer Applied Biosystems 476A protein sequencer by the method of Matsudaira.[16]

Purification of GAD

Step 1. About 90.2 g of wet cells were suspended in 1000 ml of Buffer A. The suspension was incubated with 0.2 mg/ml of lysozyme at 37°C for 15 min. After the incubation, cells were disrupted by sonication and centrifuged at 20,000 × *g* for 30 min. The supernatant was collected for further purification.

Step 2. The cell-free extract was fractionated with ammonium sulfate at 60% saturation. The precipitate was collected by centrifugation and dissolved in Buffer A.

Step 3. The sample was put on a column of Sephadex G-100 (55 × 620 mm) equilibrated with Buffer A containing 0.15 M NaCl and 5 mM EDTA. Fractions containing GAD activity were pooled and precipitated with ammonium sulfate at 80% saturation. The precipitate was collected by centrifugation and dialyzed against Buffer A at 4°C overnight.

Step 4. The dialysate was put on a column of DEAE-Sepharose CL-6B equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer and the enzyme was eluted with a 0 to 0.6 M NaCl linear gradient. Fractions containing GAD activity were pooled and dialyzed against Buffer A.

Step 5. The dialysate was put on a column of Mono Q HR 5/5 equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer and the enzyme was eluted with a 0 to 0.5 M NaCl linear gradient. Fractions containing GAD activity were pooled.

Step 6. The pooled samples were diluted two-fold with 20 mM sodium phosphate buffer, pH 7.0, and put on a column of Mono Q HR 5/5. The column conditions were the same as described in Step 5. Fractions containing GAD activity were pooled and stored at −30°C.

Results

Purification of GAD

GAD from *L. brevis* was purified by lysozyme treatment, sonication, ammonium sulfate fractionation, Sephadex G-100, DEAE-Sepharose CL-6B, and two times of Mono Q HR 5/5 column chromatographies. About 9 mg of purified enzyme was obtained from 90.2 g of wet cells. The purified enzyme showed a single protein band on SDS-PAGE with the molecular weight of 60,000 (Fig. 1). The molecular weight of purified GAD was also 120,000 by native gel filtration (data not shown). These results indicate that GAD from *L. brevis* exists as a dimer.

N-Terminal amino acid sequence

The N-terminal amino acid sequence of the purified GAD was NH_2-Met-Asn-Lys-Asn-Asp-Gln-Glu-Gln-Thr-. This sequence had no close similarity with any other proteins reported so far.

Absorption spectrum of L. brevis GAD

Figure 2 shows an absorption spectrum of GAD from *L. brevis* IFO 12005. The absorption spectrum was quiet similar to that of *E. coli* GAD.[7] From the spectrum, the molar ratio of PLP in GAD was estimated to be 0.88 mol per subunit.[7]

Effects of pH and temperature on GAD activity

When GAD activity was measured using 0.2 M pyridine–HCl buffer, the optimum activity was observed at pH 4.2 (Fig. 3A). The optimum temperature of GAD activity was at 30°C using 0.2 M pyridine–HCl, pH 4.2 (Fig. 3B). The purified GAD was incubated for 19 h at 4, 17, 30, and 37°C, then the remaining GAD activity was measured. Under these conditions, more than 90% of original GAD activities were retained below 17°C.

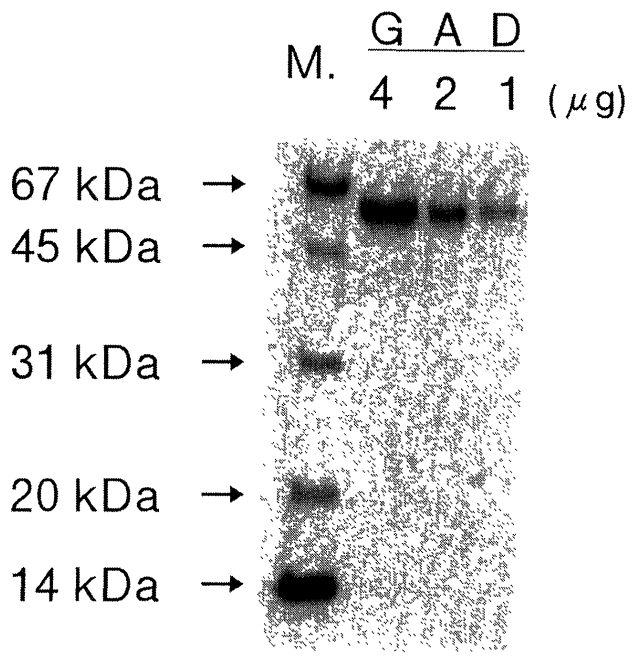

Fig. 1. SDS-PAGE of the Purified GAD from *L. brevis*.

The molecular weight of GAD from *L. brevis* was estimated with standard proteins. Bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (20,000), and lysozyme (14,000) were used as molecular weight standards. M, molecular weight standards.

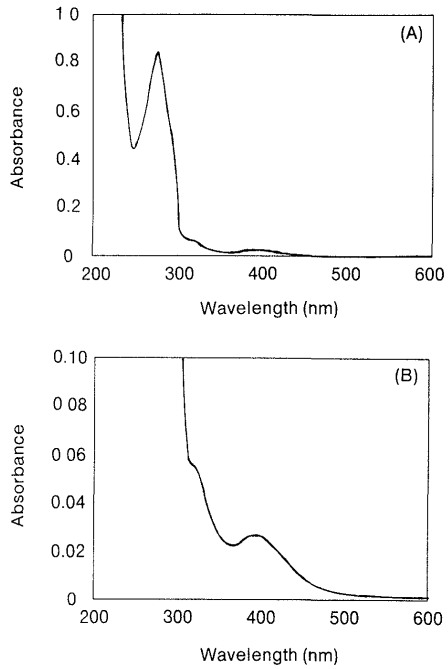

Fig. 2. Absorption Spectrum of *L. breivis* GAD.

The concentration of the enzyme was 0.367 mg/ml in 20 mM sodium phosphate buffer, pH 7.0. Absorption spectrum between 200 and 600 nm was taken from the Beckman DU-7400 diode array spectrophotometer at 1.0 (A) or 0.1 (B) full scale.

Effects of sulfate ions on GAD activity

As the GAD activity of *E. coli* has been influenced by the NaCl concentration in the reaction mixture, we tested the effects of NaCl on GAD activity of *L. brevis*. The NaCl had no effect on GAD activity of *L. brevis* (data not shown).

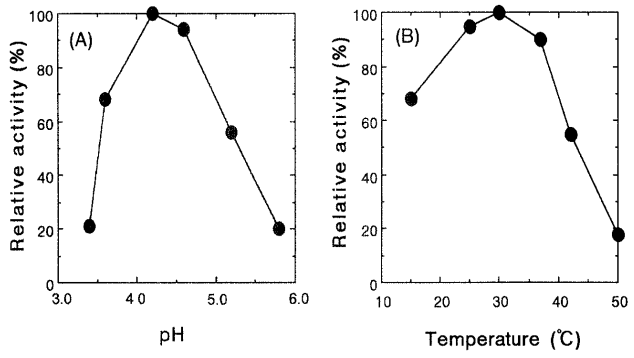

Fig. 3. Effects of pH and Temperature on GAD Activity.

(A) Optimum pH. The enzyme activity was measured in 0.2 M pyridine–HCl buffer at various pHs. (B) Optimum temperature. The enzyme activity was measured in 0.2 M pyridine–HCl, pH 4.6 at various temperatures.

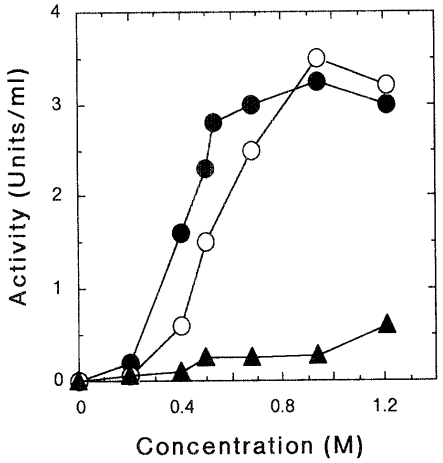

Fig. 4. Effects of Sulfate Ions on GAD Activity.

The enzyme activity was measured in 0.2 M pyridine–HCl, pH 4.6 in the presence of various concentrations of sulfates. –●–, ammonium sulfate; —○—, sodium sulfate; —▲—, magnesium sulfate.

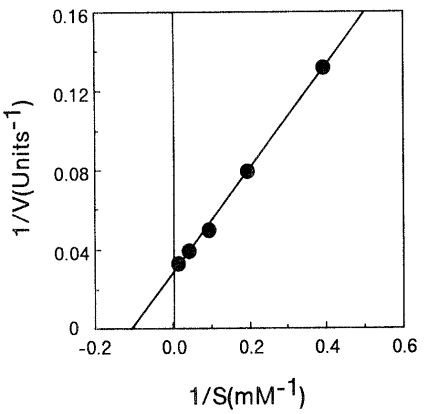

Fig. 5. Lineweaver–Burk Plot for GAD from *L. brevis*.

The enzyme was incubated with various concentrations of L-glutamic acid in 0.2 M pyridine–HCl, pH 4.6, and then the GABA concentration in the reaction mixture was measured.

We tested the effects of several ions on GAD activity and found that various sulfate ions increased GAD activity. The order of effects of sulfates were as follows: ammonium sulfate > sodium sulfate > magnesium sulfate (Fig. 4).

Substrate specificity of GAD from L. brevis

The substrate specificity of GAD from *L. brevis* was tested using 22 kinds of amino acids (L-alanine, ε-aminocaproic acid, L-arginine, L-aspartic acid, L-citrulline, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-homoserine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, and L-valine). When GAD was incubated at 37°C, and pH 4.6 for 60 min with several amino acids (20 mM), decarboxylated product was observed only for L-glutamic acid. These results indicate that GAD from *L. brevis* is specific for L-glutamic acid.

Table Properties of GADs from *L. brevis* and *E. coli*

	L. brevis[a]	*E. coli*[b]
Molecular weight		
SDS–PAGE	60,000	50,000
Gel filtration	120,000	300,000
N-Terminal sequence	M-N-K-N-D-Q-E-Q-T	M-D-Q-K-L-L-T-D-F-R-
Optimum pH	4.2	4.4
Optimum temperature	30°C	
Activation by NaCl	–	+
by $(NH_4)_2SO_4$	+	
Substrate specificity	L-Glu	L-Glu
K_m	9.3 mM	1.0 mM
k_{cat}/K_m	$7.0 \times 10^2\ M^{-1}\ s^{-1}$	

[a] This study. [b] M. L. Fonda.[8]

Kinetic parameters of the purified GAD

The kinetic parameters of GAD using L-glutamic acid as a substrate were evaluated by Lineweaver–Burk plot (Fig. 5). The K_m, k_{cat}, and k_{cat}/K_m were 9.3 mM, $6.5\ s^{-1}$, and $7.0 \times 10^2\ M^{-1}\ s^{-1}$, respectively.

Discussion

This paper is the first report of the purification and characterization of GAD from *L. breivs* IFO 12005. The purification procedure consists of lysozyme treatment, sonication, ammonium sulfate fractionation, Sephadex G-100 column chromatography, DEAE-Sepharose CL-6B column chromatography, and two cycles of MonoQ column chromatographies. The *L. brevis* cells were hard to disrupt without lysozyme treatment. The specific activity of the purified GAD was 6.0 units per mg of protein.

Some properties of GAD from *L. brevis* were compared to those of *E. coli* GAD (Table). The subunit molecular weights of GADs from *L. brevis* and *E. coli* were 60,000 and 50,000, respectively. On the other hand, the native molecular weight of *L. brevis* and *E. coli* enzymes were 120,000 and 300,000. These results indicate that GADs from *L. brevis* exist as a dimer and GAD from *E. coli* exist as a hexamer. The N-terminal amino acid sequence of GAD from *L. brevis* (NH_2-Met-Asn-Lys-Asn-Asp-Gln-Glu-Gln-Thr-) was clearly different from to that of *E. coli* enzyme (NH_2-Met-Asp-Gln-Lys-Leu-Leu-Thr-Asp-Phe-Arg-) and had no close similarity with any other protein. Therefore, structural differences might exist between *L. brevis* and *E. coli* GADs. The optimum pH and substrate specificities of GADs from *L. brevis* and *E. coli* were nearly the same. Both enzyme could not decarboxylate any amino acids except L-glutamic acid. In the case of GAD from *E. coli*, the enzyme activity was increased by the addition of NaCl. However, GAD from *L. brevis* was not increased by NaCl (data not shown). On the other hand, the *L. brevis* enzyme

activity was increased by the addition of various sulfates (Fig. 4). The order of effects were ammonium sulfate > sodium sulfate > magnesium sulfate. These results indicate that the increase of hydrophobic interaction between subunits may increase GAD activity. The K_m of GAD from *L. brevis* was 9.3 mM using L-glutamic acid as a substrate. This value is about one order of magnitude higher than that of GAD from *E. coli*.

Kimchi, fermented by *L. brevis*, has been taken by us since old times. Therefore, GAD from *L. brevis* can be applicable for a wide range of uses, *e.g.*, food, medical supplies, and other eatable products. We are now cloning the GAD gene from *L. brevis* for the detailed characterization and large scale production of *L. brevis* GAD in *E. coli*.

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