Food Sci. Technol. Res., **9** (3), 283–287, 2003

Purification and Characterization of Glutamate Decarboxylase from *Aspergillus oryzae*

Kimi TSUCHIYA,[1] Kenryo NISHIMURA[1] and Masayoshi IWAHARA[2]

[1]*Kumamoto Industrial Research Institute, Higashimachi, Kumamoto, 862-0901, Japan*
[2]*Sojo University, 4-22-1, Ikeda, Kumamoto-City, Kumamoto, 860-0082, Japan*

Received December 25, 2002; Accepted June 14, 2003

We purified glutamate decarboxylase (GAD) [EC4.1.1.15] from *Aspergillus oryzae* and characterized its biochemical and kinetic properties. GAD was purified by ammonium sulfate at 30–70% saturation and chromatographies on Sephacryl S-300, DEAE-FF and CM-FF. The purification of GAD from the crude enzyme solution was 40-fold and the recovery rate was 4.9%. About 230 μg of purified enzyme was obtained from 20 g of the mycelia of *A. oryzae*. The purified preparation of the enzyme showed a single protein band on SDS-PAGE. The molecular weight of purified GAD by SDS-PAGE and gel filtration was estimated to be 48 kDa and 300 kDa, respectively, suggesting that purified GAD had a hexameric structure. The K_m value for L-glutamic acid, a substrate of the enzyme, was estimated to be 13 mM. The optimum pH and temperature of GAD were 5.5 and 60°C, respectively. The GAD activity was stable up to 40°C.

Keywords: glutamate decarboxylase, *Aspergillus oryzae*, purification, γ-amino-butyric acid

Glutamate decarboxylase (GAD; EC4.1.1.15) produces γ-amino-butyric acid (GABA) from glutamate. GABA has several physiological effects on the human body, including neurotransmitting, hypotensive and diuretic effects. Several attempts to enrich GABA in functional foods have been reported: GABA-rich green tea produced by anaerobic treatment of green tea (Ohmori *et al*., 1987), GABA accumulation in red mold rice (Tsuji *et al*., 1992), GABA accumulation in rice germ by soaking in water (Saikusa *et al*., 1994), GABA enrichment in brown rice by high-pressure treatment (Kinefuchi *et al*., 1999) and GABA production by lactic acid bacteria (Hayakawa *et al*., 1997).

We have recently found high GAD activity in *Aspergillus oryzae* cultured in a liquid medium, and it was specially rich in *A. oryzae* used for *miso* and *sake* brewing (Tsuchiya & Nishimura, 2002a). Furthermore, we also reported that the batch reactor using pellet form mycelium of *A. oryzae* (for *miso* production), which was cultured in liquid medium achieved an extremely high GABA production rate (8.5 mmol GABA/g *Aspergillus* mycelium/h) (Tsuchiya *et al*., 2002b).

GAD is widely distributed in animate nature. In mammals, GAD has been purified from human brain (Blindermann *et al*., 1978) and mouse brain (Wu *et al*., 1973). In higher plants, GAD has been purified from squash (Matsumoto *et al*., 1986). In bacteria, the enzymatic properties of GAD have been reported in both *Escherichia coli* (Shikuya & Schwert, 1960; Fonda, 1985) and *Lactobacillus brevis* (Ueno *et al*., 1997). Although GAD obtained from *A. oryzae* has not been purified, the gene was recently cloned and the nucleotide sequence was determined (Iwai *et al*., 2001). The detailed biochemical nature of the enzyme is unclear, which we believe important in promoting the GABA production in food processing by effective utilization of the GAD from *A. oryzae*. In this study, we purified GAD from the mycelium of *A. oryzae* and examined some biochemical characteristics of the enzyme.

E-mail: tsuchiya@kmt-iri.go.jp

Abbreviations: GAD, glutamate decarboxylase; GABA, γ-amino-butyric acid; PLP, pyridoxal-5-phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GFC, gel filtration chromatography; UF, ultrafiltration.

Materials and Methods

Microorganism and the culture conditions *Aspergillus oryzae* used in this study was isolated from *tane-koji* SR-108 (Hishiroku Co., Ltd., Kyoto). *A. oryzae* used as a source of GAD was inoculated in a baffledflask (500 ml) containing 200 ml of sterilized YPD medium (4% (w/v) glucose, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4 and 0.2% $MgSO_4 \cdot 7H_2O$) and was cultured with shaking at 170 rpm for 3 days at 30°C.

Preparation of crude enzyme The mycelia of *A. oryzae* cultivated in liquid medium were washed three times with 0.9% (w/v) NaCl. The washed mycelia (fresh weight 80 g) were lysed in 50 mM phosphate buffer (pH 5.5) containing 3 mg/ml Yatalase (Takara Shuzo Co., Shiga) for 30 min at 37°C. This lysate was centrifuged for 10 min at 15,000×*g* at 4°C and the supernatant was used as crude enzyme solution.

Purification of GAD Proteins precipitated by the addition of $(NH_4)_2SO_4$ from 30% to 70% saturation were collected by centrifugation for 30 min at 15,000×*g* at 4°C. These proteins were dissolved in 10 ml of 20 mM phosphate buffer pH 5.5 (buffer A) and desalted by gel filtration using PD-10 (Amersham Biosciences Co., Ltd., Tokyo). The desalted crude enzyme solutions were put on a column of Sephacryl S-300 (φ26×600 mm, Amersham Biosciences Co., Ltd.) equilibrated with buffer A containing 0.15 M NaCl and eluted with the same buffer at a flow rate of 1 ml/min. Active fractions were collected and concentrated by UF membrane, Centricon PLUS-20 (Millipore). The concentrate was put on a Hiprep DEAE-FF column (φ16×100 mm, Amersham Biosciences Co., Ltd.) previously equilibrated with buffer A and eluted with a linear gradient of 0 to 0.6 M NaCl in this buffer. Fractions containing GAD were pooled and dialyzed

against buffer A by the same membrane. The dialysate was put on a Hiprep CM-FF column (φ16×100 mm, Amersham Biosciences Co., Ltd.) previously equilibrated with buffer A .

Enzyme assay Enzyme solution (0.1 ml) was mixed with 0.9 ml of substrate solution (100 mM phosphate buffer, pH 5.5, containing 50 mM sodium glutamate and 50 μM pyridoxal-5-phosphate (PLP)) and incubated at 37°C for 30 min. The assay reaction was stopped by the addition of 1 ml of 500 mM Na_2CO_3 solution. Then the produced GABA was measured by the method of AccQ·Tag™ by HPLC (Waters Co., Milford, MA). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA per min. Specific activity was defined as units per mg of protein. The assay of the cell-bound GAD activity was performed as follows: 0.5 g mycelium (wet weight) was mixed with 10 ml of substrate solution, and incubated at 37°C for 30 min. The enzyme reaction was stopped by eliminating the cell from the solution. Then the produced GABA in the solution was measured.

Protein assay and electrophoresis Protein concentrations were determined by the Bradford method (1976) with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Tokyo) with bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) using 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. Molecular weight standards used were SDS-PAGE standard (Bio-Rad Laboratories). Protein bands were detected by Coomassie Blue G-250 staining.

Effect of pH and temperature The optimum pH of the purified enzyme was determined with 0.1 M acetate buffer (pH 4.2 to 6.2) and 0.1 M phosphate buffer (pH 5.3 to 7.8). The effects of temperature on the purified GAD activity were examined under the standard enzyme assay conditions except that the temperature was varied from 25°C to 70°C and the reaction time was 15 min. To estimate thermal stability, the enzyme was incubated for 60 min at various temperatures (30°C to 80°C) and the residual enzyme activity was assayed under the standard assay conditions.

Results and Discussion

Solubilization of GAD from mycelia We investigated the condition for the solubilization of GAD from mycelia. GAD was hardly solubilized either by cell destruction with glass-beads (bead size 0.3 mm, Vi4, Edmund Buhler) or cell treatment with surfactant (Y-PER™, PIERCE Co., Ltd., Rockford).

Three mg/ml of Yatalase (Takara Shuzo Co., Ltd.), 3 mg/ml of lysozyme (ICN Biomedicals, Inc) and 3 mg/ml of Zymolyase-20T (ICN Biomedicals, Inc) were added to the *A. oryzae* mycelium to solubilize GAD. The GAD activity in the buffer after the solubilization treatment was expressed as a relative percentage of the cell-bound GAD activity before solubilization (Fig. 1).

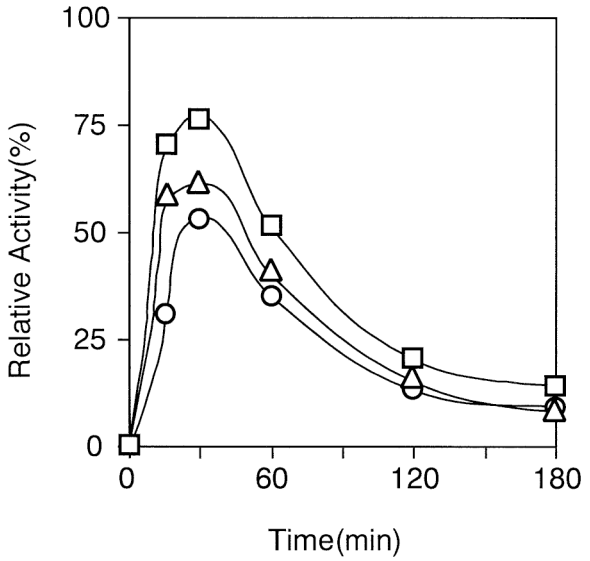

Fig. 1. Solubilization of GAD from mycelia. The mycelium of *A. oryzae* was lysed in iso-osmotic lysis buffer including 3 mg/ml of Yatalase (□), 3 mg/ml of lysozyme (△) and 3 mg/ml of Zymolyase-20T (○) with shaking and incubation at 37°C. The lysates were centrifuged at 3000×*g* for 5 min and the GAD activity of supernatants was assayed. The relative activity was expressed as the percentages of soluble activity against the whole activity of mycelia.

In each case, the GAD activity in the lysis buffer showed the maximal relative activity after 30 min of treatment, and then it decreased with incubation time until 3 h. Although the cell digestive enzyme easily solubilized GAD, it is suggested that GAD lost its enzymatic activity after solubilization. This phenomenon may be due to two different mechanisms: the loss of stability and proteolysis. The former, however, seemed to contribute much less than the latter because the soluble form GAD retained about 100% stability for up to 60 min (Fig. 5), although we have no data for 120- and 180-min incubations.

We performed 30-min incubation for the solubilization because a short period is effective to avoid the proteolysis which develops with time. Ueno *et al.* (1997) also used a short incubation for the solubilization of GAD from *L. brevis* by lysozyme.

Yatalase showed the best solubilization rate (77%) among the three enzymes.

We could not solubilize GAD by the physical cell wall destruction with glass-beads, but the treatment with digestive enzyme easily solubilized GAD from *A. oryzae*. From these findings, we speculated that *A. oryzae* GAD is present as a cell-bound enzyme, not as a cytosolic enzyme.

Enzyme purification Table 1 shows a summary of the purification steps. Crude enzyme solution contained 183.9 mg of

Table 1. Purification of the GAD from *A. oryzae*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification-fold	Recovery (%)
Crude enzyme	183.9	222.5	1.21	1.00	100.0
$(NH_4)_2SO_4$	131.2	178.5	1.36	1.12	80.2
Sephacryl S-300	11.0	81.0	7.36	6.08	36.4
DEAE-FF	0.39	11.6	29.1	24.0	5.2
CM-FF	0.23	11.0	48.2	40.0	4.9

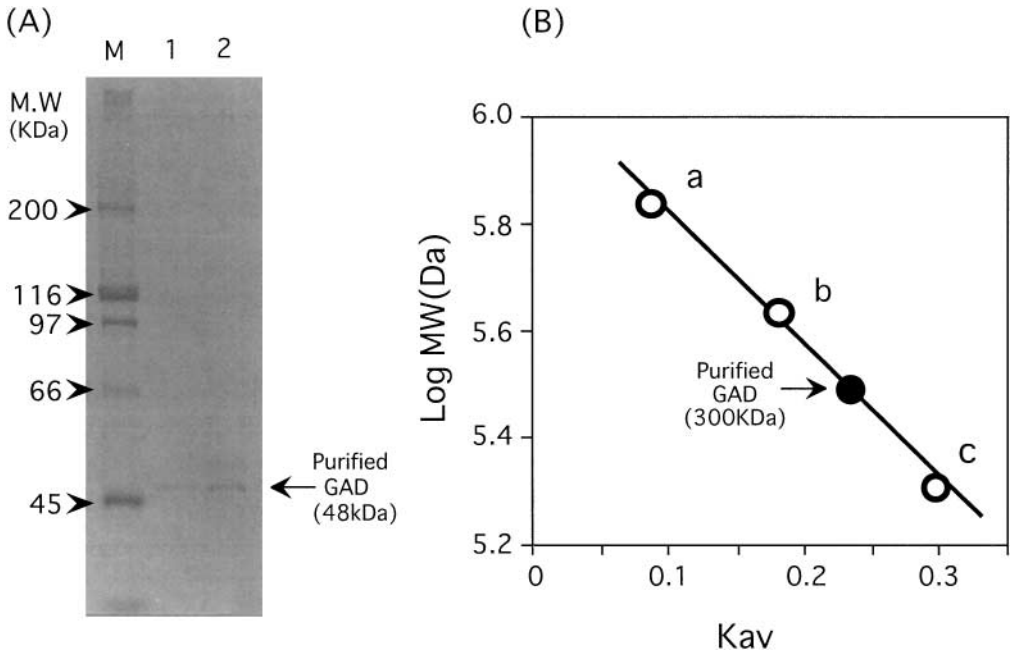

Fig. 2. SDS-PAGE and GFC of the purified GAD from *A. oryzae*. (A) SDS-PAGE was carried out on 7.5% acrylamide gel. Lane M indicates standard proteins. Myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), serum albumin (66 kDa) and ovalbumin (45 kDa) were used as molecular weight standard. Lanes 1 and 2 were the purified enzyme of 35 ng and 70 ng, respectively. (B) Plot of logarithmic molecular weight vs. K_{av} in GFC. The position of purified GAD is shown by the closed circle. The marker proteins (open circles), (a) thyroglobulin (692 kDa), (b) ferritin (437 kDa) and (c) catalase (203 kDa) are indicated.

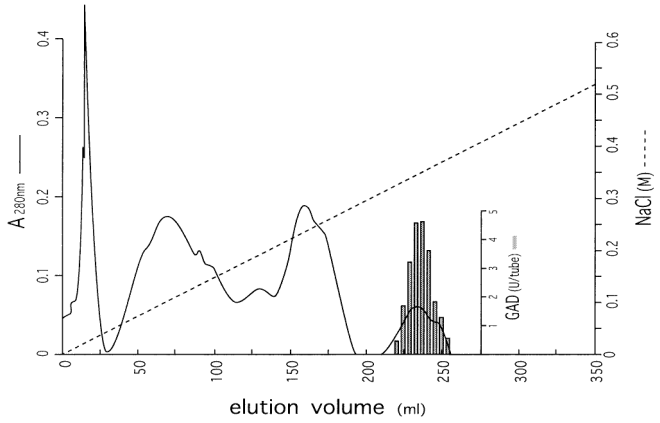

Fig. 3. Elution profiles of the protein and GAD activity using a HiPrep DEAE-FF column. Anion-exchange chromatography was done on a column (φ1.6×10 cm) of HiPrep DEAE-FF. The flow rate was 2.5 ml/min and the absorbance at 280 nm for protein was monitored. The fractions (5 ml each) of the eluate were analyzed for the enzyme activity (bar).

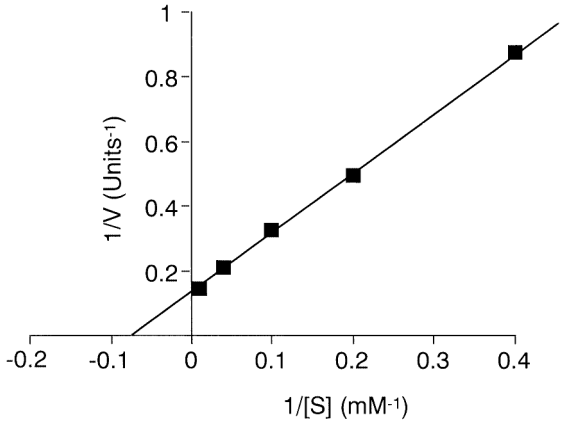

Fig. 4. Lineweaver-burk plot for GAD from *A. oryzae*. The purified enzyme was incubated with various concentrations of L-glutamic acid in 0.1 M phosphate buffer, pH 5.5.

protein and 222.5 U of GAD and $(NH_4)_2SO_4$ was added with 30–70% saturation; then gel-filtration chromatography, anion-exchange chromatography and cation-exchange chromatography were carried out for the purification. A single protein band was found on the gels in SDS-PAGE after the purification (Fig. 2). The specific activity increased 6-fold in the gel-filtration step and 4-fold increase was observed in the anion-exchange column. By adding 0.35 M of NaCl, GAD was eluted from the anion-exchange column equilibrated with buffer of pH 5.5 (Fig. 3). In this step, the recovery rate was only one seventh. This low yield could be due to the adverse effect of NaCl concentration in the elution buffer since the residual cell-bond form GAD activity decreased to 70% with 30-min incubation of 0.34 M NaCl (data not shown). Because the adverse effect was irreversible, it is suggested that the low recovery rate in this step is not due to the direct inhibition against the enzyme activity by NaCl but due to the decrease in stability of the enzyme induced by NaCl.

In the final step, non-adsorbed components in the cation-exchange column, equilibrated with buffer of pH 5.5, were separated into two peaks, and GAD was found in one peak.

The purification of GAD from the crude enzyme solution was 40-fold and 4.9% of the original activity was recovered. About 230 μg of purified enzyme was obtained from 20 g (dry weight)

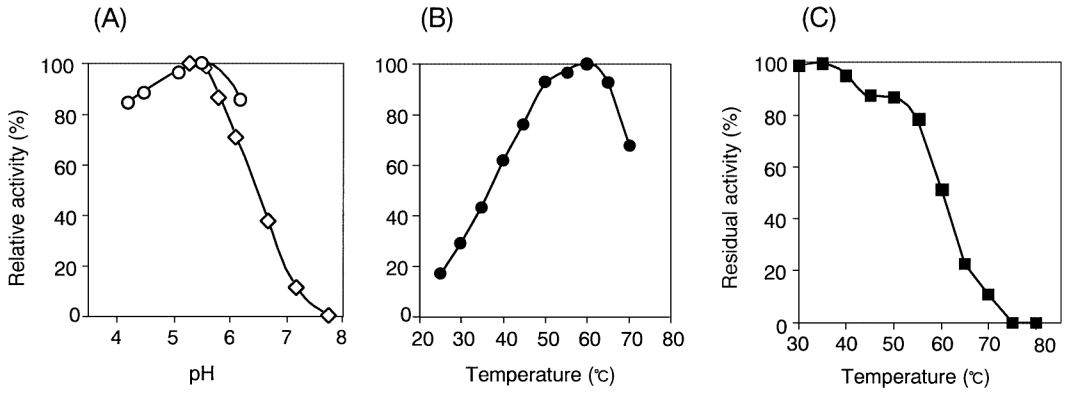

Fig. 5. Optimum pH and temperature, and heat stability of the purified GAD. (A) Optimum pH. Activity in 0.1 M acetate buffer, pH 4.2 to 6.2 (○) and 0.1 M phosphate buffer, pH 5.3 to 7.8 (◇). (B) Optimum temperature. Activity was measured in 0.1 M phosphate buffer at various temperatures (25°C to 70°C), pH 5.5. (C) Heat stability. The enzyme was incubated for 60 min at various temperatures (30°C to 80°C). The residual enzyme activity was determined under standard assay conditions.

Table 2. Comparative properties of GADs derived from several origins.

	MW (KDa)	Subunit MW (KDa)	Optimal pH	Optimal temperature (°C)	Specific activity (U/mg)	K_m for glutamate (mM)
Aspergillus oryzae	300	48	5.5	60	48.2	13.3
Lactobacillus brevis	120	60	4.2	30	6.0	9.3
Escherichia coli	300		3.8			0.8
Escherichia coli	310	50	4.5		67.9	1.0
Squash	340	58	5.8	60	25.8	8.3
Human brain	140	67	6.8		1.0	1.3

of the mycelia of *A. oryzae*. After the final step of the purification, the specific activity increased to 48.2 U/mg, which is twice that of GAD from squash (Matsumoto *et al*., 1986) and 6 times that of GAD from *L. brevis*, but lower than that of GAD from *E. coli* (Fonda, 1985) (Table2).

Molecular weight and structure of GAD The molecular weight of GAD was estimated to be approximately 48 kDa by SDS-PAGE, whereas the native molecular weight was estimated as approximately 300 kDa by Sephacryl S-300 gel filtration (Fig. 2). This suggests that this enzyme is a hexamer. As shown in Table 2, the molecular weight of GAD purified from *A. oryzae* was almost the same as that from *E. coli* (Shikuya & Schwert, 1960; Fonda, 1985) and similar to that from squash (Matsumoto *et al*., 1986). These enzymes had hexameric structure. On the other hand, the GADs derived from human (Blindermann *et al*., 1978), mouse (Wu *et al*., 1973), rat (Blindermann *et al*., 1978) and *L. brevis* (Ueno *et al*., 1997) were reported to be dimer.

K_m and V_{max} value of GAD To examine the affinity of GAD to the substrate, the concentration of L-glutamic acid was changed from 2.5 mM to 100 mM, and the K_m and V_{max} value of the enzyme was calculated. The kinetic parameters of GAD for the substrate were evaluated by Lineweaver-Burk plot (Fig. 4). The K_m and V_{max} value of the GAD was calculated to be 13.3 mM and 7.2 units at pH 5.5 at 37°C. When the value is compared with those from other organisms, the K_m value of GAD from *E. coli* was 0.8 mM (Shikuya & Schwert, 1960) and 1.0 mM (Fonda, 1985), and that of GAD from *L. brevis* was 9.3 mM (Ueno *et al*., 1997). From these findings, it can be concluded that the affinity to the substrate of GAD from *A. oryzae* is less than that of GAD from *L. brevis*, and one tenth that of GAD from *E. coli*. The V_{max}/K_m value which is an indicator of reaction efficacy for GAD from *A. oryzae* was calculated as 5.4×10^2 U·M^{-1}.

Effect of pH and temperature on GAD activity Figure 5 shows that the optimum pH of GAD was 5.5 (A) and its optimum temperature was 60°C (B), which was determined after 15-min. reaction. Figure 5 (C) shows the heat stability of GAD after 60-min incubation. It is suggested that GAD was stable below 40°C because the residual activities were more than 94% at those temperatures. On the other hand, GAD lost 50% of its activity at 60°C in spite of that being the optimum temperature.

Table 2 shows comparisons of the properties of GADs purified from *A. oryzae* and those from other organisms. In optimum pH and temperature, GAD from *A. oryzae* was more similar to that from squash than to that from *E. coli*. When we compared the GAD from *A. oryzae* with that from *L. brevis*, the molecular weight, enzyme structure, optimum pH and temperature were quite different. These properties of GAD purified from *A. oryzae* and human are also different.

The strain of *A. oryzae* used in this study for the purification of GAD is in common use in the *miso* industry, and is widely accepted to have a safe origin. In the previous study, we reported the usefulness of the reactor using this strain of *A. oryzae* in the production of GABA (Tsuchiya *et al*., 2002b), and also showed that it could be applied to enrich the GABA content in liquid food (Tsuchiya *et al*., 2003). In this study, we identified the biochemical characteristics of GAD of *A. oryzae* in an attempt to maximize GABA production of the reactor.

References

Blindermann, J.M., Maitre, M., Ossola, L. and Mandel, P. (1978). Purification and some properties of L-glutamate decarboxylase from human brain. *Eur. J. Biochem*., **86**, 143–152.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Fonda, M., L. (1985). L-Glutamate decarboxylase from bacteria. *Methods Enzymol.*, **113**, 11–16.

Hayakawa, K., Ueno,Y., Kawamura, S., Taniguchi, R. and Oda, K. (1997). Production of γ-aminobutyric acid by lactic acid bacteria. *Seibutsu-Kogaku Kaishi*, **75**, 239–244 (in Japanese).

Iwai, Y., Kato, Y., Furukawa, K., Hara S. (2001). Japan Kokai Tokkyo Koho, P2001-54390A, Feb. 27.

Kinefuchi, M., Sekiya, M., Tamazaki, A. and Yamamoto, K. (1999). Accumulation of GABA in brown rice by high pressure treatment. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **46**, 323–328 (in Japanese).

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Matsumoto, T., Yamaura, I. and Funatsu, M. (1986). Purification and properties of glutamate decarboxylase from squash. *Agric. Biol. Chem.*, **50**, 1413–1417.

Ohmori, M., Yano, T., Okamoto, J., Tsuchida, T., Murai, T. and Higuchi, M. (1987). Effect of anaerobically treated tea on blood pressure of spontaneously hypertensive rats. *Nippon Nogeikagaku kaishi*, **61**, 1449–1451 (in Japanese) .

Saikusa, T., Horino, T. and Mori, Y. (1994). Accumulation of γ-aminobutyric acid (Gaba) in the rice germ during water soaking. *Biosci. Biotechnol. Biochem.*, **58**, 2291–2292.

Shikuya, R. and Schwert, G.W. (1960). Glutamic acid decarboxylase. *J. Biol. Chem.*, **235**, 1649–1652.

Tsuchiya, K. and Nishimura, K. (2002a). Characterization of glutamate decarboxylase (GAD) in mold for brewing. *Nippon Jozo Kyokaishi*, **97**, 382–386 (in Japanese).

Tsuchiya, K., Nishimura, K. and Iwahara, M. (2002b). Production of γ-aminobutyric acid (GABA) by *Aspergillus oryzae* and the separation of GABA using electric dialysis. *Nippon Jozo Kyokaishi,* **97**, 878–882 (in Japanese).

Tsuchiya, K., Nishimura, K., Matsuda, T. and Iwahara, M. (2003). Enrichment of γ-amino butyric acid (GABA) in *shochu* post-distillation slurry using a continuous-flow type stirring reactor with *Aspergillus oryzae*. *Nippon Jozo Kyokaishi,* **98**, 132–138 (in Japanese).

Tsuji, K., Ichikawa, T., Tanabe, N., Abe, S., Tarui, S. and Nakagawa, Y. (1992). Antihypertensive activities of *Beni-Koji* extracts and γ-aminobutyric acid in spontaneously hypertensive rats. *Eiyougaku Zasshi*., **50**, 285–291 (in Japanese).

Ueno, Y., Hayakawa, K., Takahashi, S. and Oda, K. (1997). Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci. Biotechnol. Biochem.*, **61**, 1168–1171.

Wu, J.Y., Matsuda, T. and Roberts, E. (1973). Purification and characterization of glutamate decarboxylase from mouse brain. *J. Biol. Chem.*, **248**, 3029–3034.