PROPERTIES OF GLUTAMATE DECARBOXYLASE (GAD) FROM EDIBLE MUSHROOM

NORIFUMI SHIRASAKA†, TAKAHIRO YOSHIDA, KAZUKO IWAMOTO, TAKAO TERASHITA
Laboratory of Food Microbiological Science and Biotechnology
Division of Applied Biological Chemistry, Graduate School of Agriculture, Kin-ki University
3327-204, Nakamachi, Nara 631-8505
Japan
sirasaka@nara.kindai.ac.jp

ABSTRACT

Enzymatic properties of glutamate decarboxylase (GAD) [EC4.1.1.15] obtained from the fruiting body of *Flammulina velutipes* were evaluated via protein isolation, purification, and characterization. To investigate the anatomical localization of GAD, crushed fruiting bodies were centrifuged; the supernatant and precipitate were subsequently subjected to enzyme reaction. GAD activity of the precipitate was much stronger than that of the supernatant. Although Nonidet P40 slightly solubilized GAD protein from cell wall fractions, most of the activity remained in the cell debris. A nearly homogeneous protein band was observed in SDS-PAGE analysis after 10 treatments of solubilization and subsequent purification with ammonium sulfate precipitation and ultrafiltration. In cell wall-binding enzyme experiments, formation of γ-amino butyric acid (GABA) was observed between pH 4 and 6, and the maximum GAD activity was observed at pH 6. However, GAD activity was lost after overnight dialysis against buffering of pH 6-10. The enzyme activity was optimum at 28°C and stable below 50°C. GAD of *F. velutipes* was specific for L-glutamate.

Key words: glutamate decarboxylase (GAD), *Flammulina velutipes*, γ-amino-butyric acid (GABA)

INTRODUCTION

Glutamate decarboxylase (GAD; EC4.1.1.15) is a pyridoxal enzyme and produces γ-amino-butyric acid (GABA) from glutamate. GABA and GAD are widely distributed in mammals [1, 2], plants [3], and microorganisms [4-9]. 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 [10], GABA accumulation in red mold rice [11], GABA accumulation in rice germ by soaking in water [12], GABA enrichment in brown rice by high-pressure treatment [13] and GABA production by lactic acid bacteria [14].

The major enzymatic properties of GAD have been previously described [1-3, 5, 6, 9, 15]. Although many edible mushrooms accumulate GABA in their fruiting body, only a few studies have reported mushroom-derived enzyme.

In this study, we investigated the purification and enzymatic properties of *Flammulina velutipes* GAD.

MATERIALS AND METHODS

Materials. Fruiting bodies of *F. velutipes* used in this study were purchased in local supermarkets in Nara prefecture, Japan and stored frozen until use.

Crude enzyme preparation. Frozen fruiting bodies were cut into small pieces (ca. $1cm^3$) and crushed with 50 mM phosphate buffer (pH 5.5) containing 1 mM pyridoxal-5'-phosphate (PLP) and 1mM phenylmethylsulfonyl fluoride (PMSF). This homogenate was centrifuged for 30 min at 15,000 × *g* at 4°C. The resultant supernatant and cell debris were used as a crude enzyme source of GAD after dialysis.

Enzyme assay. Enzyme solution (0.1 ml) was mixed with 0.9 ml of substrate solution (50 mM phosphate buffer, pH 5.0, containing 10 mM sodium glutamate and 50 □M PLP) and incubated overnight at 28°C. The enzyme reaction was stopped by eliminating the cells from solution. Then, the formation of GABA was checked by thin-layer chromatography. The activity of GAD was estimated semi-quantitatively by color spot intensity.

Solubilized GAD enzyme preparation. Cell wall debris was obtained as precipitate of crushed and centrifuged fruiting bodies. The precipitate was washed 3 times with twice the volume of 50 mM phosphate buffer (pH 5.5) containing 1 mM PLP and 2% Nonidet P40. GAD enzyme was solubilized by washing 5 times with twice the volume of the same buffer. Solubilized enzyme solutions were combined and subsequently precipitated with ammonium sulfate precipitation at 70% saturation. Isolated proteins were dissolved in a small volume of 20 mM phosphate buffer (pH 5.5) and subjected to concentration by UF membrane.

SDS-PAGE analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [16] using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS. Molecular weight standards used were SDS-PAGE standards (Bio-Rad Laboratories), and protein bands were detected by Coomassie Blue G-250 staining.

pH and temperature analysis. The optimum pH for enzyme activity was determined with 0.05 M acetate buffer (pH 4.0 and 5.0), 0.05 M phosphate buffer (pH 6.0 to 7.0), 0.05 M Tris-HCl buffer (pH 8.0 to 9.0) and 0.05 M borate buffer (pH 10.0). The effect of pH on enzyme stability was elucidated by dialysing the enzyme overnight at 4°C in the same buffers described above. The effects of temperature on GAD activity were examined under standard enzyme assay conditions except that the temperature was varied from 4°C to 60°C and the reaction time was overnight. To estimate thermal stability, the enzyme was incubated for 30 min at various temperatures (4°C to 70°C), and the residual enzyme activity was assayed under the standard assay conditions.

RESULTS AND DISCUSSION

Localization and solubilization of GAD. To clarify enzyme localization within the fruiting bodies, GAD activity of both supernatant and cell debris was assayed. When cell debris was used as an enzyme source, strong GAD activity was observed (Fig. 1). In contrast, supernatant fraction did not show apparent GABA spots. This result demonstrated that *F. verutipes* GAD is a cell wall-binding enzyme. A similar cell wall binding type of GAD was reported in *Aspergillus oryzae* [6]. In the case of *Aspergillus*, cell wall-degrading enzyme mixtures (Yatalase and lysozyme) effectively solubilized the GAD

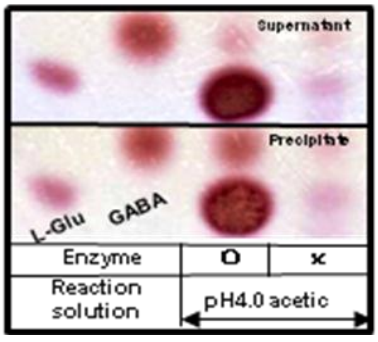

Figure 1. Localization of GAD activity.
The reaction was carried out in 50 mM acetate buffer (pH4.0) with 10 mM sodium glutamate and 50 μM pyridoxal-5'-phosphate (PLP)) at 37°C for overnight.

enzyme from cell walls. However, cell wall-degrading enzyme treatments with Yatalase were not effective for *F. velutipes* GAD because apparent GAD activity was not detected in Yatalase-treated supernatant.

In order to solubilize GAD activity, several detergents and cell-degrading enzymes were tested. Although Nonidet P40 (Sigma) was the best reagent for solubilization, its efficiency was not robust. However, Nonidet P40 was utilized because it was the most effective solubilizer at the time of this study.

For GAD enzyme solubilization, cell debris was treated with 50 mM phosphate buffer (pH 5.5) containing 1 mM PLP and 2% Nonidet P40. This procedure was repeated 5 times, and resulting supernatants were pooled as enzyme solution. A large part of GAD activity remained in cell wall fractions after solubilization treatment procedures. Crude enzyme proteins were precipitated from combined solution by ammonium sulfate with 30–70% saturation. The precipitated protein pellet was dissolved in a small amount of 20 mM phosphate buffer (pH 5.5) and subjected to concentration by UF membrane.

Enzyme purity and molecular weight of GAD. To check the purity of solubilized GAD, UF-concentrated samples were subjected to SDS-PAGE analysis. A single major protein band and several minor bands were observed (Fig. 2). The molecular weight of this major band, presumably GAD, was estimated to be approximately 30 kDa.

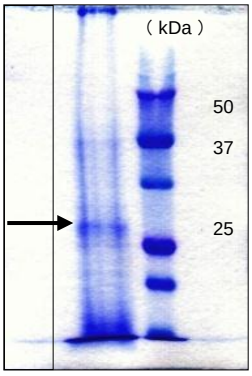

Figure 2. SDS-PAGE analysis.
The arrow shows the band of presumed GAD.

Enzymatic properties of GAD. GAD protein was the most abundant protein bound to cell wall fractions after treatment with Nonidet P40. This result led to the selection of cell debris as an enzyme source for pH, temperature, and substrate specificity evaluation.

Figure 3 depicts the effect of pH on GAD activity. GAD activity was detected at pH 4 to pH 6, and GABA spot color intensity was not different within this range. However, GAD activity was lost after dialysis in the buffer at pH 6. Effects of temperature are shown in Figure 4. GAD activity was detected between 4-60°C. Color spot intensity was highest at 28°C, stable below 37°C, and slightly decreased at 60°C.

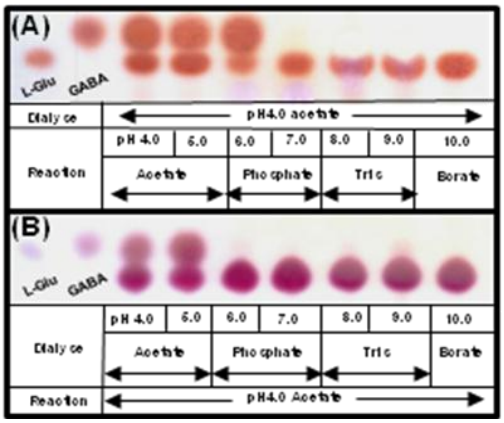

Figure 3. Effect of pH on enzyme activity.
The optimum pH (A) of the enzyme was determined with each indicated buffer. The effect of pH on enzyme stability (B) was elucidated by dialysing the enzyme overnight at 4°C in the indicated buffers.

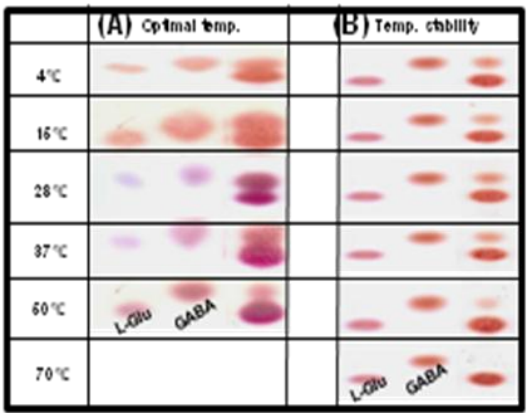

Figure 4. Effect of temperature on enzyme activity.
The effects of temperature (A) on the GAD activity were examined under the standard enzyme assay conditions at indicated temperatures. For thermal stability (B), the enzyme was incubated for 30 min at indicated temperatures and the residual enzyme activity was assayed under the standard assay conditions.

To elucidate enzyme substrate specificity, several α-amino acids listed in Figure 5 were added to the reaction mixture. If α-decarboxylation occurred, correspondent products would be detected. However, L-glutamate was a specific substrate for GAD; therefore, no other substrate was used (Fig. 5)

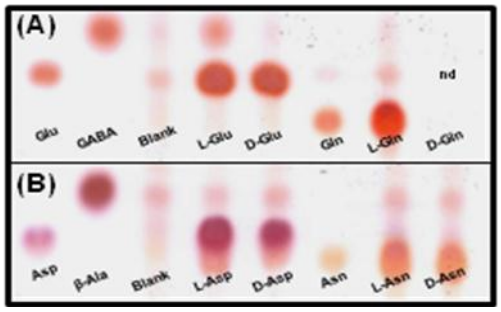

Fig. 5. Substrate specificity.
The enzyme was incubated with the indicated amino acid in 50 mM acetate buffer at 28°C

Table 1. Comparative properties of GADs derived from several origins.

	MW (KDa)	Subunit MW (KDa)	Opt. pH	Opt. Temp. (°C)	Spec. Act. (U/mg)	*K*m for L-glu (mM)	Ref
Flammulina verutipes	nd	30	4 - 6	28	nd	nd	This study
Aspergillus oryzae	300	48	5.5	60	48.2	13.3	[6]
Lactobacillus brevis	120	60	4.2	30	6.0	9.3	[5]
Escherichia coli	300	-	3.8	-	-	0.8	[4]
Escherichia coli	310	50	4.5	67.9	-	1.0	[7]
Squash	340	58	5.8	60	25.8	8.3	[3]
Human brain	140	67	6.8	-	1.0	1.3	[1]

Table 1 shows comparisons between GADs partially purified from *F. velutipes* and those from other organisms. At optimum pH and temperature, GAD from *F. velutipes* was more similar to that from *L. brevis.* The molecular weight of *L. brevis* GAD lesser than that of all GAD proteins listed in Table 1.

CONCLUSION

GAD was partially purified from *F. vertipes*, and its enzymatic properties were elucidated. *F. vertipes* GAD was strongly bound to cell walls and difficult to solubilize. Although Nonidet P40 could slightly solubilize GAD protein from cell wall fractions, most of the activity remained in the cell debris. A nearly single protein band was observed in SDS-PAGE analysis after 10 solubilization treatments and subsequent purification procedure with ammonium sulfate precipitation and ultrafiltration. Cell wall-binding enzyme experiments revealed GABA formation between pH 4 and 6, with the maximum GAD activity occurring at pH 6. However, GAD activity was lost after overnight dialysis against buffered at pH ranging from 6–10. The enzyme activity was optimum at 28°C and stable below 50°C. GAD of *F. velutipes* was specific for L-glutamate.

REFERENCES

[1] Blindermann J.M., *et al.* (1978). Purification and some properties of L-glutamate decarboxylase from human brain. *Eur. J. Biochem.* 86: 143-152.
[2] Wu J.Y., Matsuda T., Roberts E. (1973). Purification and characterization of glutamate decarboxylase from mouse brain. *J. Biol. Chem.* 248: 3029-3034.
[3] Matsumoto T., Yamaura I., Funatsu M. (1986). Purification and properties of glutamate decarboxylase from squash. *Agric. Biol. Chem.* 50: 1413-1417.

[4] Fonda M.L. (1985). L-Glutamate decarboxylase from bacteria. *Methods Enzymol.* 113: 11-16.
[5] Ueno Y., *et al.* (1997). Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci. Biotechnol. Biochem.* 61: 1168-1171.
[6] Tsuchiya K., Nishimura K., Iwahara M. (2003). Purification and Characterization of Glutamate Decarboxylase from *Aspergillus oryzae*. *Food Sci. Technol. Res.* 9: 283-287.
[7] DeBiase D, *et al.* (1996). Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid decarboxylase from *Escherichia coli*. *Protein Expr Purif* 8: 430-438.
[8] Baldy P. (1975). Metabolism of γ-aminobutyrate in *Agaricus bisporus*. I. L-Glutamate-1-carboxylase. *Physiol. Plantarum* 34: 365-372.
[9] Liu W.H., Wang C.L. (2002). Culture conditions and some properties of glutamate decarboxylase produced by *Monascus purpureus*. *Food Sci. Agric. Chem.* 4: 15-19.
[10] Ohmori M., *et al.* (1987). Effect of anaerobically treated tea on blood pressure of spontaneously hypertensive rats. *Nippon Nogeikagaku kaishi, (in Japanese)* 61: 1449-1451.
[11] Kono I., Himeno K. (2000). Changes in γ-aminobutyric acid content during bebi-koji making. *Biosci. Biotechnol. Biochem.* 64: 617-619.
[12] Saikusa T., Horino T., Mori Y. (1994). Accumulation of γ-aminobutyric acid (Gaba) in the rice germ during water soaking. *Biosci. Biotechnol. Biochem.* 58: 2291-2292.
[13] Kinefuchi M., *et al.* (1999). Accumulation of GABA in brown rice by high pressure treatment. *Nippon Shokuhin Kagaku Kogaku Kaishi, (in Japanese)* 46: 323-328.
[14] Hayakawa K., *et al.* (1997). Production of γ-aminobutyric acid by lactic acid bacteria. *Seibutsu-Kogaku Kaishi, (in Japanese)* 75: 239-244.
[15] Hiraga K., Ueno Y., Oda K. (2008). Glutamate Decarboxylase from *Lactobacillus brevis*:Activation by Ammonium Sulfate. *Biosci. Biotechnol. Biochem.* 72: 1299-1306.
[16] Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.