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Purification and characterization of glutamate decarboxylase from rice germ

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Abstract

Glutamate decarboxylase (EC 4.1.1.15, GAD) is a pyridoxal 5′-phosphate (PLP) dependent enzyme, which catalyses the irreversible α-decarboxylation of L-glutamic acid to γ-aminobutyric acid. GAD was purified 186-fold from rice germ using a combination of ammonium sulfate fractionation, DEAE-Sepharose FF ion exchange chromatography, Superdex-200 gel filtration chromatography, and Glu-Sepharose CL 4B affinity chromatography. The purified preparation showed a single peak on SE-HPLC with an approximate molecular mass of 78 kDa and a single band on SDS–PAGE with a subunit Mr of 40 kDa. This indicated that the GAD from rice germ existed as a dimer of homological subunits. Rice germ GAD has an optimum pH range between 5.5 and 5.8, and an optimum temperature at 40 °C. K_m values for glutamic acid and PLP were determined at 32.3 mM and 1.7 μM, respectively. Chemicals reagents such as $HgCl_2$, KI and $AgNO_3$ decreased the enzyme activity by 68.5%, 44.9% and 32.4%, respectively, but 500 μM of $CaCl_2$ at the optimum pH could increase the activity by 145%.

Keywords: Rice germ; Glutamate decarboxylase; Purification; Characterization

1. Introduction

Glutamate decarboxylase (GAD) (EC 4.1.1.15) is an important enzyme in biological metabolisms. It catalyzes the conversion of L-glutamic acid (Glu) to γ-aminobutyric acid (GABA) and carbon dioxide by α-decarboxylation. GAD is widely distributed in nature and found in abundance in animals, higher plants, and microorganisms. In mammals, GAD has been purified from brains of human (Blindermann, Maitre, Ossola, & Mandel, 1978), pig (Spink, Porter, Wu, & Martin, 1985), and mouse (Wu, Matsuda, & Roberts, 1973). GAD has been used as an antigen for the diagnosis and prediction of insulin-dependent diabetes mellitus (IDDM) (Zimmet, 1996). The most intensively researched GAD is from bacteria *Escherichia coli* (Biase, Tramonti, John, & Bossa, 1996), by which the physio-chemical properties of GAD have been examined in detail. The GAD from *E. coli* has also been used to detect pathogenic *E. coli* groups in water and food (Grant, Stephen, & Peter, 2001; Rice, Johnson, Dunnigan, & Reasoner, 1993). In addition, GAD has been purified from lactic bacteria and used to produce GABA-enriched food (Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998; Nomura et al., 1999; Ueno, Hayakawa, Takahashi, & Oda, 1997). In fungi, GAD was purified and characterized from *Aspergillus oryzae* (Tsuchiya, Nishimura, & Iwahara, 2003) and *Neurospora crassa* Conidia (Hao & Schmit, 1991). In higher plants, GAD was purified and characterized from squash (Matsumoto, Yamaura, & Funatsu, 1986; Matsumoto, Yamaura, & Funatsu, 1996), potato (Satyanarayan & Nair, 1985), cowpea (Johnson, Singh,

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Cherry, & Locy, 1997), wheat (Fan, Li, Zhu, & Chen, 1998), and barley (Inatomi & Slaughter, 1975). Research on GAD indicated that GAD activity could be affected by a variety of stress-related conditions, including anaerobiosis (Tsushida & Murai, 1987), cytosolic pH reduction (Crawford, Bown, Breitkreuz, & Guinel, 1994), cold-stress (Naidu, Paleg, Aspinall, Jennings, & Jones, 1991), and heat-stress (Mayer, Cherry, & Rhodes, 1990).

The earliest study of GAD in rice was initiated in 1964 by Bautista, Lugay, Cruz, and Juliano (1964), who found that GAD activity was a more reliable index for the viability of different stored rice. Later in 1994, Saikusa, Horino, and Mori (1994a, 1994b) found that water soaking of rice kernel under a slightly acidic condition resulted in a remarkably increased level of GABA content, which indicated that the GAD of rice was more likely in the germ fraction. Based on this discovery, an efficient and simple method via water soaking has been developed (Ohtsubo, Asano, Sato, & Matsumoto, 2000; Saikusa et al., 1994b) for production of GABA from rice germ in an effort to develop novel functional food for hypertension prevention. Akama, Akihiro, Kitagawa, and Takaiwa (2001) isolated cDNA clones encoding two distinct GADs and their genomic clones from rice. However, there is at this moment no research published about the characterization of GAD from rice. To the best of our knowledge, the previous researches of rice GAD focused on its application on GABA production and there was no information available of the purification and characterization of the rice GAD. The rice GAD can be better used to produce GABA enriched food after its properties have been more clearly known. Therefore in this paper we report the purification and some properties of the GAD from rice germ.

2. Materials and methods

2.1. Materials

Rice Germ was generously provided by Shanghai Store & Transport of Grain Co., Ltd. (Shanghai, China), and stored in refrigerator at −4 °C until analysis. Resins of DEAE-Sepharose Fast Flow, Superdex 200 and CNBr Sepharose CL 4B were purchased from Pharmacia Biotech (Uppsala, Sweden). Standard proteins (e.g., rabbit phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43 kDa; bovine carbonic anhydrase, 31 kDa; trypsin inhibitor, 20.1 kDa; hen egg white lysozyme, 14.4 kDa) for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and size exclusion-high pressure liquid chromatography (SE-HPLC) were obtained from Beijing Jinke Biotech (Beijing, China) and used as molecular mass standards. Protein KW802.5 (8 × 300 mm, 5 μm) GFC column was obtained from Shodex Company (Kawasaki, Japan). L-Glutamic acid, GABA, phenylmethylsulfonylfluoride (PMSF) and pyridoxal 5′-phosphate (PLP) were purchased from Sigma Chemical Co (St. Louis, USA). All other chemicals were in reagent or higher grade. Standard buffer was prepared in 50 mM sodium phosphate buffer at pH 5.8 containing 0.2 mM PLP and 1 mM PMSF.

2.2. Determination of GAD activity

The reaction mixture consisted of 200 μL of 50 mM sodium phosphate, pH 5.6, 100 mM L-glutamate, 0.2 mM PLP, and 100 μL of enzyme liquid. The reaction solution was incubated at 40 °C for 60 min, and then terminated by addition of 100 μL of 32% (w/v) trichloroacetic acid (TCA). The suspension was filtered through a 0.45-μm membrane filter (Whatmann, USA). The filtrate was analyzed for its GABA content by Agilent 1100 HPLC (Agilent Technologies, USA) (Zhang, Wu, & Yao, 2003). One unit of GAD activity was defined as release of 1 μmol of GABA produced from glutamate per 30 min at 40 °C. Specific activity was defined as units of GAD activity per mg of the enzyme.

2.3. Protein assay

Protein concentrations in various preparations were assayed by the method of Bradford (1976).

2.4. Preparation of crude GAD

Two hundred grams of the rice germ were homogenized at 10 000 rpm for 15 min with 1000 mL of 50 mM sodium phosphate buffer, pH 5.8, containing 0.2 mM PLP, and 2 mM 2-mercaptoethanol (ME), 2 mM EDTA, and 1 mM PMSF. After homogenization, the protein extract was filtered through four layers of cheesecloth and centrifuged at 7000*g* for 20 min at 4 °C. Solid ammonium sulfate was then added to this crude extract up to 30% saturation and centrifuged at 10 000*g* for 20 min at 4 °C. The supernatant was adjusted to 50% saturation by further addition of solid ammonium sulfate and centrifuged again at 10 000g for 20 min at 4 °C. The precipitate was dissolved in 100 mL of the standard buffer. The mixture was dialyzed three times against 2 L of the standard buffer. The supernatant of the dialysate was called the crude GAD and used for further purification.

2.5. DEAE-Sepharose FF ion exchange chromatography

A 1.6 × 50 cm DEAE-Sepharose FF ion exchange column was equilibrated with the standard buffer at 4 °C. Three millilitres of the crude GAD were loaded onto the open column. GAD was eluted with a linear gradient of 0–1.4 M NaCl in the standard buffer at 4 °C. Those fractions with more than 60% of GAD specific activity were pooled and concentrated by ammonium sulfate precipitation (70% saturation). The precipitate was dissolved in and dialyzed against the standard buffer.

2.6. Superdex-200 gel filtration chromatography

The dialyzed enzyme pool was loaded on a 1.0 × 100 cm Superdex-200 column, pre-equilibrated with the standard buffer. The column was washed with two volumes of the same buffer at 4 °C. The fractions with relatively high GAD specific activities were pooled together. The enzyme pool was concentrated by ammonium sulfate precipitation (70% saturation), dissolved in, and dialyzed against the standard buffer.

2.7. Glu-Sepharose CL 4B affinity chromatography

Glu-Sepharose CL 4B was prepared by the method of Fan, Li, Li, Ji, and Lu (2001) with some modifications. The copper salt of L-glutamate (50 mg) was dissolved in 100 mL of 0.5 M NaCl solution. Then the solution was adjusted to pH 8.0. Five grams of CNBr-activated Sepharose CL-4B resin was added to the solution and the mixture was stirred for 5 h at room temperature. The resulting gel was then packed in the column (1.0 × 30 cm) and treated with 250 mL of 0.05 M of EDTA to remove copper (II) that was bound to the α-amino carboxyl terminals of the gel as complexes. The eluant was adjusted to pH 8.0 with 0.1 M of NaOH and made up to 300 mL with water. The Cu (II)–EDTA complexes were measured using a colorimetric determination at 730 nm. From the amount of copper (II) eluted by this treatment, the amount of L-glutamate bound to the gel was estimated to 1.7 μmol of gel. The gel was washed with 500 mL of a 0.5 M solution of NaCl, and equilibrated with the standard buffer at 4 °C. The enzyme with GAD activity separated from Superdex-200 was loaded on the Glu-Sepharose CL 4B column. The bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in the standard buffer. Fractions containing GAD activity were pooled, dialyzed against the standard buffer and concentrated by ultra filtration (PM 10 membrane, Pall Corporation, USA) at 4 °C.

2.8. Polyacrylamide gel electrophoresis

SDS–PAGE was carried out on a 0.75 mm thick slab gel with a 15% (w/v) resolving gel (Laemmli, 1970). Protein samples with GAD activity were treated with 0.25% (w/v) 2-ME and 1% (w/v) SDS prior to being loaded on the gel.

2.9. Measuring apparent molecular mass of GAD by SE-HPLC

Twenty microlitres of the purified GAD containing 10 μg of the protein were loaded on the protein KW802.5 column. Then the enzyme was eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl. The flow rate was 0.5 mL/min and absorbance was monitored at 280 nm. Twenty microlitres of the protein standards were also loaded on the same column and eluted by the same way. Molecular mass of GAD was estimated by calibrating the column with protein standards.

2.10. Determination of K_m for glutamate and PLP

The K_m value for glutamic acid was determined by varying the concentration of glutamic acid from 28.6 mM to 200 mM with constant 0.2 mM PLP in the standard assay mixture. Prior to determining the K_m of PLP, the enzyme solution was dialyzed against the 50 mM sodium phosphate buffer, pH 5.6, for 24 h at 4 °C to remove the free PLP. The K_m determination of PLP was performed in the standard assay mixture with concentrations of PLP varied from 2.0 μM to 12 μM at constant 100 mM glutamic acid. The K_m values were estimated by using double reciprocal via the Lineweaver–Burk plot.

2.11. pH dependence and pH stability

A pH-dependent activity profile of the purified GAD was determined using two buffer systems (0.5 M citrate buffer, pH 2.5–4.5; and 0.5 M sodium phosphate buffer, pH 5.0–8.0). pH stability was determined after mixing GAD with buffers at various pHs using three buffer systems (0.5 M citrate buffer, pH 2.5–4.5; 0.5 M sodium phosphate buffer, pH 5.0–8.0 and 0.5 M Tris–HCl buffer, pH 8.5–9.0) One millilitre of the enzyme solution was mixed with 4.0 mL of 0.5 M buffer in various pHs and left at 4 °C for 10 h. The mixture was then carefully adjusted to pH 5.6. The remaining enzyme activity was measured as those described above.

2.12. Temperature dependence and thermal stability

A temperature-dependent activity profile of the purified GAD was determined using different temperatures. For the thermal stability of GAD, GAD solution (pH 5.6) was left at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C, respectively, for 1 h, then the remaining enzyme activity was measured as those described above.

2.13. Effect of various reagents on GAD activity

Fifty microlitres of various chemical reagents (2 mM) including KCl, KI, $MgSO_4$, $MnSO_4$, $Al_2(SO_4)_3$, $AgNO_3$, $LiSO_4$, $HgCl_2$ and $CaCl_2$, as well as 100–700 μM $CaCl_2$ were incubated with 50 μL of the enzyme liquid (0.2 mg/ml) at 30 °C for 30 min. The remaining GAD activity was measured as those described above.

2.14. Statistical analysis

Mean values of listed data were average of triplicates. The effects of various reagents on the activity of the purified enzyme were subjected to analysis of variance using Microsoft Excel. The least significant difference was used

to find the significant effect of various reagents on GAD activity at $P \leqslant 0.05$.

3. Results and discussion

3.1. Purification of the enzyme

This paper is the first report of the purification and characterization of GAD from rice germ. The purification procedures consisted of ammonium sulfate precipitation and chromatography techniques as outlined above in Section 2. Enzymatic activities of the GAD eluants from DEAE-Sepharose FF, Superdex-200, and Glu-Sepharose CL 4B are shown in Figs. 1–3, respectively. These purification steps resulted in 186.2-fold purification of GAD from rice germ (Table 1). Moreover, about 1.2 mg of GAD was obtained from 10 g of rice germ with a 12.6% yield. The specific activity of the purified GAD was 223.4 U per mg of protein.

Although the GAD has been reported in purification without using affinity chromatography for several kinds of plant GAD (Fan et al., 1998; Johnson et al., 1997; Matsumoto et al., 1986), we were unable to obtain a pure GAD only using ion-exchange and gel filtration chromatography repeatedly even after three to four times. Additionally, we could not obtain the purified GAD only using the Glu-Sepharose CL 4B affinity chromatography either, though Satyanarayan and Nair (1985) purified the potato GAD by only one step of the Glu affinity chromatography. Therefore, the rice germ GAD was finally purified by those three combined chromatographic techniques mentioned above.

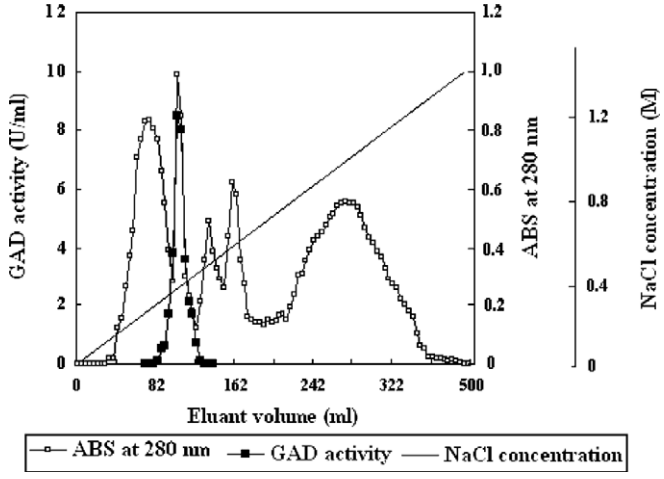

Fig. 1. The elution of GAD from DEAE-Sepharose FF chromatography.

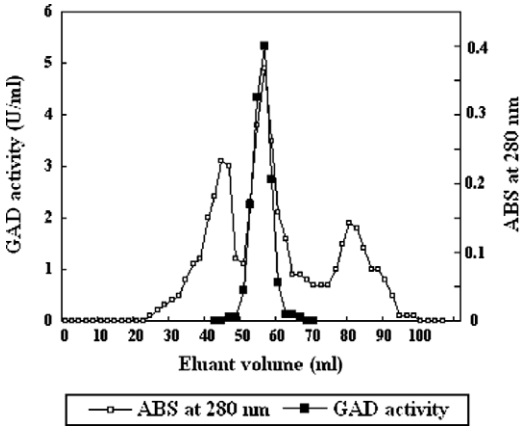

Fig. 2. The elution of GAD from Superdex 200 chromatography.

3.2. Homogeneity of purified enzyme

The homogeneity of the purified GAD was examined by SE-HPLC (data not shown) and SDS–PAGE (Fig. 4). The SE-HPLC gave a single sharp peak from the purified protein with an apparent molecular mass of 78 kDa. A single band was observed on SDS–PAGE with the subunit Mr of 40 kDa. These results suggested that the rice germ GAD had two homological subunits. The molecular mass of rice germ GAD was very similar with the potato GAD. Satyanarayan and Nair (1985) found that the potato GAD displayed two homological subunits at 45.5 kDa. However, from rice shoot cDNA library, Akama et al. (2001) isolated full-length cDNAs for two distinct isoforms

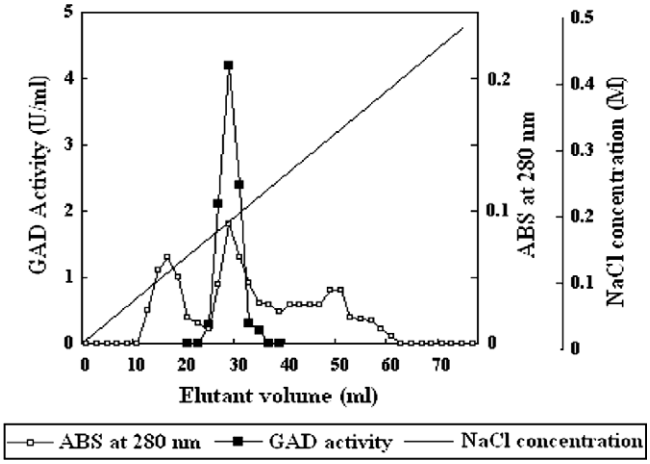

Fig. 3. The elution of GAD from Glu Sepharose CL 4B affinity chromatography.

Table 1
Purification of GAD from rice germ

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude GAD	1770.0	2124.0	1.2	1.0	100
DEAE-Sepharose FF fraction	45.7	1293.3	28.3	23.6	60.1
Superdex 200 fraction	7.6	994.8	130.9	109.1	46.8
Glu Sepharose CL 4B fraction	1.2	268.1	223.4	186.2	12.6

Results are means of at least three determinations.

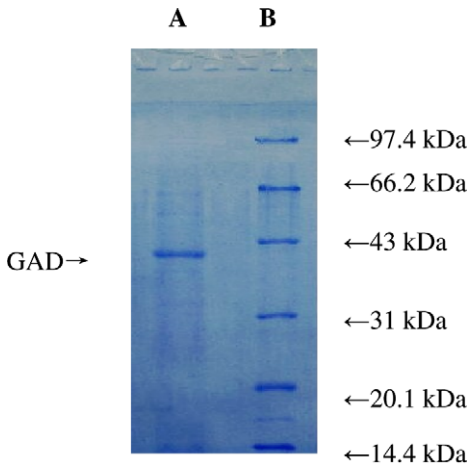

Fig. 4. SDS–PAGE of purified GAD. Line A, 2 μg of purified GAD; line B 10 μg of molecular standards (from top to bottom: rabbit phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43 kDa; bovine carbonic anhydrase, 31 kDa; trypsin inhibitor, 20.1 kDa; hen egg white lysozyme, 14.4 kDa).

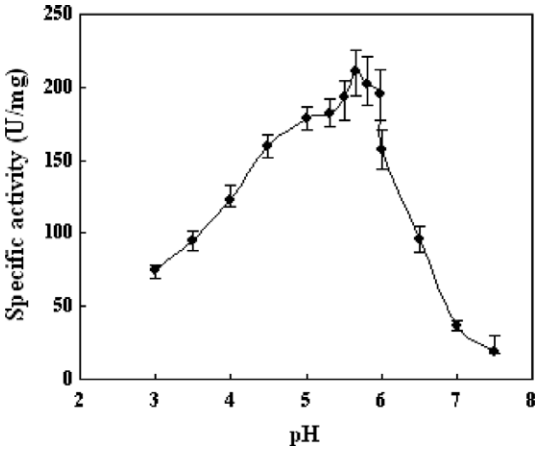

Fig. 5. Effect of pH on GAD activity. Buffers used were: 0.5 M citrate buffer, pH 2.5–4.5; and 0.5 M sodium phosphate buffer, pH 5.0–8.0 ($n = 3$).

of GAD. Open reading frames found in these two cDNAs encode putative proteins of 56.7 kDa and 55.6 kDa. So the apparent molecular mass of the purified GAD from rice germ was higher than the putative mass of the GADs from rice shoot cDNA. In addition, the native rice germ GAD seemed to have no other forms. It indicates that the GADs obtained by different methods and from the different part of rice have the different molecular structure. The other purified plant GADs have been found in different forms. For example, barley embryos GAD had two forms including a 256 kDa form and a 120 kDa form, and barley root GAD was a single species with an Mr of 310 kDa (Inatomi & Slaughter, 1975). Squash GAD consisted of multiple identical subunits of 58 kDa (Matsumoto et al., 1986).

3.3. Determination of K_m and V_{max}

As determined by the linear Lineweaver–Burk plot of $1/V$ against 1/[S] on the purified GAD, the values of K_m and V_{max} for glutamic acid were 32.3 mM and 11.2 μmol mg^{-1} min^{-1}, respectively. The values of K_m and V_{max} for PLP were 1.7 μM and 11.3 μmol mg^{-1} min^{-1}, respectively. The K_m value of the rice germ GAD for glutamic acid of 32.3 mM is larger than that of GADs of other plants, such as squash (8.3 mM), cowpea (3.2 mM), potato (5.6 mM), barley embryos (3.1 mM) and barley root (22 mM). Also, this value is about 1–2 orders of magnitude higher than that of GAD from *E. coli* and human brain (Matsumoto et al., 1986).

3.4. Effect of pH and temperature

The optimum pH of the rice germ GAD was between 5.5 and 5.8 and maximally active at pH 5.6 (Fig. 5). The enzyme was stable within the pH range of 4.5–8.0 (data not shown). The remaining activity was about 40% and 20% at pH 4.0 and 9.0, respectively. The optimum pH values of the purified GAD from bacteria, plants, and animals differ from each other. The optimum pHs are 3.5–5.0 among the bacteria GAD, 5.5–6.5 among the plant GAD, and about 6.8–7.0 among the animal GAD (Matsumoto et al., 1986; Satyanarayan & Nair, 1985). Nevertheless, the optimum pH and temperature of the rice germ GAD were quite similar to other plant GAD (Inatomi & Slaughter, 1975; Satyanarayan & Nair, 1985; Fan et al., 1998; Johnson et al., 1997; Matsumoto et al., 1986; Matsumoto et al., 1996). The activity of the GAD was optimal at 40 °C (Fig. 6). The optimum temperatures of most plants GAD are between 37 °C and 40 °C (Satyanarayan & Nair, 1985; Fan et al., 1998; Inatomi & Slaughter, 1975), except for squash GAD at 60 °C. The enzyme was also stable at the temperatures between 0 °C and 50 °C (data not shown), but it was fairly unstable at 50 °C and above. Eighty-seven percent of the GAD activity was lost at 60 °C.

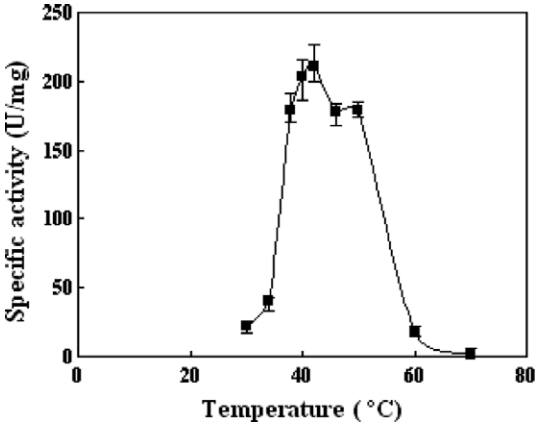

Fig. 6. Effect of the temperature on GAD activity. That was measured at various temperatures in the buffer consisting of 200 μL of 50 mM sodium phosphate (pH 5.6), 100 mM L-glutamate, 0.2 mM PLP ($n = 3$).

3.5. Effect of chemical reagents on GAD activity

As shown in Table 2, the reagents did not significantly inactivate the enzyme except $HgCl_2$, KI, and $AgNO_3$ that decreased the enzyme activity by 68.5%, 44.9% and 32.4%, respectively. The same effect of $HgCl_2$ on the rice GAD activity was found in brown rice during water soaking (Liu, Zhai, & Wan, 2005). Since KI rather than KCl significantly decreased the GAD activity, it seemed that the ion I^- instead of Cl^- had inhibited the enzyme. On the other hand, various tested sulfates, such as $MgSO_4$, $MnSO_4$, $Al_2(SO_4)_3$ and $LiSO_4$, had no effect on the rice germ GAD activity. On the contrary, activity of the GAD from *Lactobacillus brevis* could be significantly increased by the addition of various sulfates in an order of effect as follows: ammonium sulfate > sodium sulfate > magnesium sulfate. The enzyme activity was influenced by sulfate ions in a dose-dependent manner (Ueno et al., 1997). Such difference in respondence to the same ions might be due to the structural difference between the plant GAD and the bacterial GAD.

Moreover, plant GAD has been found to be able to bind calmodulin (CaM) (Baum, Chen, Arazi, Takatsuji, & Fromm, 1993; Snedden, Koutsia, Baum, & Fromm, 1996). The soybean GAD could be stimulated by 2–8-folds in the presence of calcium/calmodulin at its optimum pH (Snedden, Arazi, Fromm, & Shelp, 1995). The cowpea GAD in its crude and partially purified preparations was found to be able to be activated by the calcium/calmodulin (Johnson et al., 1997). In addition, calcium/calmodulin enabled to stimulate the accumulation of GABA in the rice roots (Aurisano, Bertani, & Reggiani, 1995). Akama et al. (2001) discovered that one putative GAD cDNA clone contained a CaM-binding domain (CaMBD). The GAD might also be a CaM-binding protein, stimulated by Ca^{2+} and CaM. In this study, we also found that the rice germ GAD activity was significantly affected by the Ca^{2+}. As shown in Fig. 7, the GAD exhibited a maximal value of its relative activity at 145% at the optimum pH after the addition of 500 μM of $CaCl_2$. This result corroborated that the rice germ GAD was regulated by the calcium/calmodulin complex.

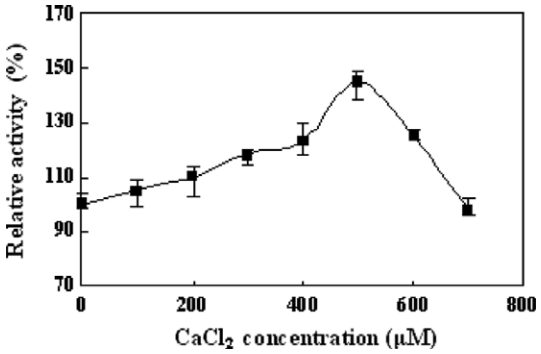

Fig. 7. Effect of $CaCl_2$ on GAD activity. Fifty microlitres of 100–700 μM $CaCl_2$ were incubated with 50 μL of the enzyme liquid (0.2 mg/ml) at 30 °C for 30 min. The activity in the absence of $CaCl_2$ was taken as 100% ($n = 3$).

Considering the fact that rice germ is a by-product in rice processing and a large quantity of rice germ is commercially available, it seems there will be no restrictions for developing cost-effective rice germ GAD-related functional food for a wide range of perspectives as dietary supplement and/or nutraceuticals against hypertension, sleeplessness, depression, and autonomic disorder (Tadashi et al., 2000). Our studies including the effect of pH and temperature on GAD activity and the stimulation of GAD by $CaCl_2$ are helpful for comprehensive utilization of rice germ to produce GABA enriched food.

Table 2
Effect of chemical reagents on GAD activity

Reagent	Relative activity (%)
Control[a]	100
KCl	84.7 ± 2.6
KI	55.1 ± 1.4[b]
$MgSO_4$	99.2 ± 2.7
$MnSO_4$	97.4 ± 3.0
$Al_2(SO_4)_3$	95.7 ± 2.7
$AgNO_3$	68.6 ± 1.5[b]
$LiSO_4$	96.6 ± 3.8
$CaCl_2$	97.8 ± 3.9
$HgCl_2$	31.5 ± 2.8[b]

The GAD activity was the average of three replicates twice analyzed.
[a] The activity of the control in the absence of chemical reagents was taken as 100%.
[b] $p < 0.05$.

Acknowledgments

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