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ScienceDirect
Food Chemistry 105 (2007) 65–71
Food Chemistry
www.elsevier.com/locate/foodchem

Some molecular properties of glutamate decarboxylase from rice germ

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Received 1 November 2006; received in revised form 13 December 2006; accepted 16 March 2007

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 (GABA). GAD was purified 186-fold from rice germ. Ultraviolet–visible spectra showed that the rice germ holoGAD presented a weak peak at 420 nm, but the inactivated apoGAD did not. The holoGAD also exhibited a strong peak at 308 nm and a weak peak at 336 nm in its fluorescence emission spectrum. The apoGAD led to a 20% increase in the fluorescence emission at 308 nm. The contents of the secondary structure elements of the holoGAD and apoGAD were estimated from the values of the mean residue ellipticity based on the CD spectra. The holoGAD had a greater β-sheet content than the apoGAD (39% versus 27%), whereas both had a similar α-helix content (13% versus 14%). These findings confirmed that a slight conformational change had occurred when PLP bound to the apoGAD to form the holoGAD. Chemical modifications of the GAD by some selected reagents indicated that histidine residue(s) might be involved in the enzymatic functions, but were not essential for the enzyme activity. The study also suggested there was one arginine residue in the GAD active site, and most likely at the substrate-binding site.

Keywords: Rice germ; GAD; Glutamate decarboxylase; GABA; Pyridoxal phosphate; Chemical modification

1. Introduction

Glutamate decarboxylase (L-glutamate-1-carboxylyase, 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 (GABA) and carbon dioxide. This enzyme exists ubiquitously in eukaryotes and prokaryotes, but its presence varies in different types of different organisms.

In mammalian brain, GAD is present in at least two forms, named GAD65 and GAD67 (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991). Both of them require the cofactor PLP for their activities (Robert, 1995). The intact GAD-cofactor complex is called holoenzyme (holoGAD). Once the cofactor is removed, the remaining protein, called apoenzyme (apoGAD), will lose its enzyme activity. ApoGAD serves as a reservoir that can be activated by binding PLP when additional GABA synthesis is required (Martin & Rimvall, 1993). The intermediate complex involves an ionic interaction between the phosphate group of PLP and the positive, charged residues on the active site of GAD, to maintain the PLP in an appropriate position in the active centre (Chen, Wu, & Martin, 1998). Chemical modifications of mammalian brain GAD by sulfhydryl reactive reagents resulted in a great weakening of the GAD activity, which suggested that cysteine residue(s) might play an important role in GAD function (Wu & Roberts, 1974). Wei and Wu (2005)

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doi:10.1016/j.foodchem.2007.03.036

recently confirmed that cysteine 446 in the holoGAD was present with a free sulfhydryl group that was important for its enzymatic activity.

GAD isoforms (GadA and GadB) have been reported in some bacterial species, including the Gram-negative bacterium *Escherichia coli* (Smith, Kassam, Singh, & Elliott, 1992) and the Gram-positive bacterium *Listeria monocytogenes* (Cotter, Gahan, & Hill, 2001). The catalytic contribution of residue lysine (Lys 276) in the active-site of *E. coli* GAD has been confirmed, with evidence showing that mutation, of Lys 276 could make the protein less flexible and its active site less accessible to the substrate and cofactor (Tramonti, John, Bossa1, & Biase, 2002). It was demonstrated that *E. coli* GAD was naturally localised exclusively in the cell's cytoplasm, but tended to move to the cell's membrane when environmental pH fell (Capitani et al., 2003).

Among the currently discovered GADs from different origins, only plant GAD has been found to be able to bind calmodulin (CaM) (Baum, Chen, Arazi, Takatsuji, & Fromm, 1993; Snedden, Koutsia, Baum, & Fromm, 1996), which is essential for the GAD's regulation of GABA and glutamate metabolisms. This regulation is critical for a plants' normal development (Baum et al., 1996). In fact, plant GAD can be activated in response to stresses such as hypoxia, temperature shock, darkness and mechanical trauma (Shelp, Bown, & McLean, 1999). These stresses can lead to the accumulation of GABA and eventually stimulate the tricarboxylic acid (Krebs) cycle. Considering the GAD's important role in GABA regulation, previous research on plant GAD has been mainly focused on the effect of CaM binding to the enzyme. GADs from petunia (Baum et al., 1993; Chen, Baum, & Fromm, 1994), soybean (Sendden, Arazi, Fromm, & Shelp, 1995), fava bean (Ling, Snedden, Shelp, & Assmann, 1994), tomato (Gallego, Whotton, Picton, Grierson, & Gray, 1995), and tobacco (Yun & Oh, 1998) have all been shown to have a CaM-binding domain at their C-terminus, but such a domain is lacking in bacterial and animal GADs.

Compared with the extensive and intensive molecular and structural investigations of bacterial and animal GADs (Chen et al., 1998; Wu & Roberts, 1974; Wei & Wu, 2005; Tramonti et al., 2002; Capitani et al., 2003), there are few reports available on plant GADs so far, especially on monocotyledonous plants. Kishinami and Ojima (1980) reported that the GABA level in cultured rice cells can be raised after adding ammonium or glutamine. Reggiani, Cantú, Brambilla, and Bertani (1988) found accumulation of GABA in rice roots under anoxia. Also, calcium and calmodulin were found to be able to stimulate the accumulation of GABA in rice roots. This suggested that GAD activity in the rice cell was modulated by calcium/calmodulin (Aurisano, Bertani, & Reggiani, 1995). Furthermore, Akama, Akihiro, Kitagawa, and Takaiwa (2001) isolated cDNA clones encoding two distinct GADs and their genomic clones from rice, and confirmed that one GAD contained a CaM-binding domain (CaMBD). The same research group also found that gene expression was differentially regulated in roots and maturing seeds. In addition, Saikusa, Horino, and Mori (1994) found that the GABA content in the rice germ increased remarkably after water soaking under slightly acidic conditions. This indicated that the GAD of rice was most likely in the germ fraction. Based on this finding, Ohtsubo, Asano, Sato, and Matsumoto (2000) developed an efficient and simple method for GABA production using rice germ, which prompted the utilisation of rice germ for functional foods.

In our previous studies, we have purified GAD from rice germ and found that it was formed in two homological subunits with approximately equivalent subunit masses of 40 kD. Rice germ GAD has an optimum pH range between 5.5 and 5.8, and an optimum temperature of 40 °C. Its K_m values for glutamic acid and PLP were determined at 32.3 mM and 1.7 μM, respectively (Zhang, Yao, Chen, & Wang, 2007). However, other molecular properties of rice germ GAD are still not clear. In this paper, some molecular characteristics of rice germ GAD are reported.

2. Materials and methods

2.1. Materials

Rice germ was generously provided by Shanghai Grain Store and Transport Co., Ltd., and stored at −4 °C before analysis. Resins of DEAE-Sephrose Fast Flow (FF), Superdex 200 and CNBr Sephrose CL 4B were purchased from Pharmacia Biotech. L-Glutamic acid, GABA, PLP, phenylmethylsulphonylfluoride (PMSF), *N*-bromosuccinimide (NBS), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), diethylpyrocarbonate (DEPC), 2,3-butanedione (BD), iodoacetic acid (IAA), chloramines-T (ch-T), and 4-morpholineethanesulfonic acid (MES) were purchased from Sigma Chemical Co. All other chemicals were reagent grade. Standard buffer was prepared in 50 mM phosphate buffer at pH 5.8 containing 0.2 mM PLP and 1 mM PMSF.

2.2. Determination of GAD activity

The assay reaction volume consisted of 200 μl containing 50 mM sodium phosphate, (pH 5.6), 30 mM L-glutamate, 20 mM PLP, and 100 μl of liquid enzyme. The reaction mixture was incubated at 40 °C for 60 min, and terminated by addition of 100 μl of 32% (w/v) trichloroacetic acid (TCA). The suspension was filtered through a 0.45-μm membrane filter (Whatman). The filtrate was analysed for its GABA content by an Hitachi 835 amino acid analyser (Hitachi). One unit of GAD activity was defined as the release of 1 μmol of GABA produced from glutamate per 30 min at 40 °C.

2.3. Purification of GAD from rice germ

GAD was purified from rice germ by the methods of Zhang et al. (2007).

2.4. Protein assay

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

2.5. Preparation of holoGAD and apoGAD solutions

HoloGAD obtained from the purified enzyme solution was further dialysed against 50 mM sodium phosphate buffer, (pH 5.6), for 24 hrs at 4 °C, to remove the free PLP. To obtain apoGAD, holoGAD solution was added to concentrated aspartate solution to give a final concentration of 5 mM, and incubated at 4 °C for about 30 min, and then dialysed against 50 mM sodium phosphate buffer, (pH 5.6), containing 2 mM MES, 2 mM EDTA, and 1 mM PMSF, to replace the inherent PLP and the excessive amount of aspartate. To reconstitute holoGAD, PLP solution at a final concentration of 10 μM was added to purified apoGAD. In these experiments, additions of PLP or aspartate solutions did not significantly dilute the enzyme concentration because the added concentrated PLP or aspartate solutions were in small volumes.

2.6. Ultraviolet and visible spectra

The spectra of GAD were obtained using a UV-1100 spectrophotometer (Beijin Ruili Analytical Instrument Company, China). Enzyme samples (0.1 mg/ml) were kept in ice until the spectrophotometrical analysis was performed. UV/visible spectra were recorded in 50 mM phosphate buffer at pH 5.6.

2.7. Fluorescence emission intensity

Fluorescence emission intensities of holoGAD and apoGAD were measured according to the methods of Chen et al. (1998) and Wei and Wu (2005). The enzyme samples were analysed in 50 mM phosphate buffer at pH 5.6 on a RF-5301 luminescence spectrometer (Shimadzu, Japan). GAD solutions (0.03 mg/ml) were excited at either 280 nm or 295 nm, and the emission spectra were recorded from 290 nm to 450 nm. Comparable measurement of a black control was determined in 50 μM PLP in 50 mM phosphate buffer at pH 5.6.

2.8. Circular dichroism (CD)

CD measurements for GAD were performed using a Jasco J-715 spectrophotometer (Jasco, Japan). The spectrum of each GAD sample (0.2 mg/ml) prepared in 50 mM phosphate buffer at pH 5.6 was measured from 190 to 300 nm, with a scanning rate of 10 nm/min with an average of five scans. Computer software supplied by Jasco was employed to operate the instrument and perform noise reduction, unit conversion, and data analysis. The secondary structure of GAD was estimated from the CD data using the same computer program.

Table 1
Effects of group-selective reagents on GAD activity

Reagent[a]	[Reagent] (mM)	Group selectivity[b]	Inactivation (%)	Buffer[c]
NBS	50	Trp	<5	A
DEPC	60	His	40	B
BD	10	Arg	100	C
ch-T	50	Met	<10	D
PMSF	50	–OH	<5	B
EDC	50	–COOH	<5	B
IAA	50	–S–S–	<10	B

[a] Phenylmethylsulfonylfluoride (PMSF), N-bromosuccinimide (NBS), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), diethylpyrocarbo-nate (DEPC), 2,3-butanedione (BD), iodoacetic acid (IAA), chloramines-T (ch-T).

[b] The most likely side chains modified under the conditions of the experiments.

[c] A: 100 mM acetate buffer, pH 4.0; B: 100 mM phosphate buffer, pH 7.0; C: 100 mM Mes buffer, pH 5.0; D: 100 mM phosphate buffer, pH 7.5. Results are means of at least three determinations.

2.9. Chemical modification experiments

Selective chemical modifications of the reactive groups of GAD were performed at 30 °C for 30 min by adding 25 μl of respective reagents to the 50 μl enzyme solution (0.2 mg/mL). The reagents and the buffers for the reactions were given in Table 1. In detail, *N*-bromosuccinimide (NBS) was selected to modify tryptophan, diethylpyrocarbonate (DEPC) for histidine, 2,3-butanedione (BD) for arginine, chloramines-T (ch-T) for methionine, phenylmethylsulphonylfluoride (PMSF) for hydroxyl groups, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) for carboxyl group, and iodoacetic acid (IAA) for disulfide bond. After each modification, the reaction was terminated by adding of 750 μl of 50 mM sodium acetic buffer, (pH 5.0). Then, one hundred microlitres of the mixture was taken for the determination of the remaining GAD activity.

2.10. Substrate protection

Prior to the chemical modification of GAD by BD, 50 ml of GAD (0.4 mg/ml) was incubated with 50 μl of 10 M glutamate in 10 mM sodium phosphate, pH 7.5, at 30 °C for 5 min.

2.11. Statistical analysis

Average values of triplicates were calculated. The data obtained from the studies were analysed using linear or quadratic regression.

3. Results and discussion

3.1. UV and visible spectra

The spectrum of holoGAD in 50 mM sodium phosphate buffer, pH 5.6, exhibited two peaks, a strong absorptive

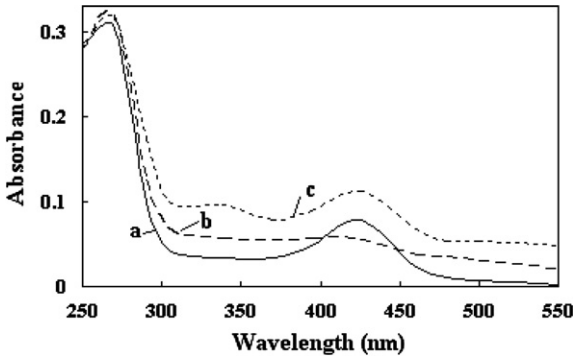

Fig. 1. UV and visible spectra of GAD. (a) holoGAD; (b) apoGAD and (c) apoGAD + 10 μM PLP. The concentration of GAD is 0.1 mg/ml in 50 mM sodium phosphate buffer, pH 5.6.

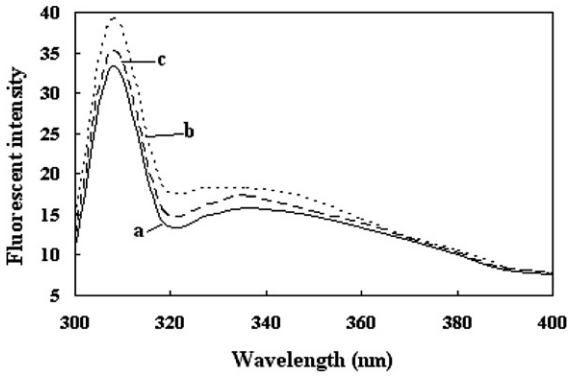

Fig. 2. Fluorescence emission spectra of GAD. (a) holoGAD; (b) apoGAD; (c) apoGAD + 10 μM PLP. The concentration of GAD is 0.03 mg/ml in 50 mM sodium phosphate buffer, pH 5.6.

peak near 280 nm and another weak peak at 420 nm (curve a in Fig. 1). This spectral profile is different from the previously reported spectra of animal and bacterial GADs. The brain GAD presented an additional peak at 332 nm besides the protein absorbance at 280 nm and the Schiff base formation at 422 nm (Chen et al., 1998). The GADs from *Lactobacillus brevis* (Ueno, Hayakawa, Takahashi, & Oda, 1997) and *Escherichia coil* (Tramonti et al., 2002) have very similar spectra with two peaks around 330 nm and 420 nm. The 420 nm peak represents the ketoenamine tautomer and is usually assigned to the Schiff base formation, but the peak responsible for absorption at 330 nm has not been clearly elucidated. Spectra of plant GADs seemed to be quite different. The potato GAD exhibited a maximum absorption at 360–370 nm in sodium phosphate buffer at its optimum pH 5.8 (Satyanarayan & Nair, 1985). Squash GAD was reported to have a similar spectrum to rice germ GAD with an absorptive peak at 420 nm (Matsumoto, Yamaura, & Funatsu, 1996). In addition, we observed that the spectrum of rice GAD was subject to pH changes from pH 3.0–8.0, and its absorbance at 420 nm reached a maximal value at pH 5.6–6.0. This pH-dependent spectral change has also been observed in other GADs (Tramonti et al., 2002; Satyanarayan & Nair, 1985). When PLP was removed from rice holoGAD to form apoGAD, the weak peak at 420 nm disappeared (curve b in Fig. 1). This peak could be restored when 10 μM of PLP was added to reconstitute the holoGAD (curve c in Fig. 1). This confirms that PLP is involved in binding the active site of GAD to form the Schiff base.

3.2. Fluorescence emission spectra

The holoGAD in 50 mM sodium phosphate buffer, pH 5.6, exhibited a strong fluorescence emission peak at 308 nm and a weak yet broad peak spanning from 320 to 380 nm (maximum emission at 336 nm) when excited at 295 nm (curve a in Fig. 2). In contrast, removing PLP from the holoGAD to form the apoGAD led to a 20% increase in the fluorescence emission intensity at 308 nm. Meanwhile, there was no significant change in the emission intensity of the fluorescence peak at 336 nm (curve b in Fig. 2). However, adding 10 μM PLP to the apoGAD quenched the fluorescence increase to its original level in the holoGAD. Nevertheless, whether removing or adding PLP, GAD's maximum fluorescence (λ_{max}) remained completely unchanged at 308 nm (curve c in Fig. 2). Similar spectral changes of fluorescence emission intensity were observed when the holoGAD and apoGAD were excited at 285 nm (data not shown). Minor changes of the fluorescence emission in 308 nm shown in Fig. 2 indicated that the microenvironment around the tryptophan residue in the GAD had been changed when PLP was removed from the holoGAD, but that change was not significant. These changes also implied that interaction of PLP with rice germ GAD might have caused a small conformational change in the enzyme. By contrast, brain GAD only exhibited one peak in its fluorescence emission spectra, and its spectral changes were similar to that of rice germ GAD when PLP was removed from and added back into the GAD (Chen et al., 1998).

3.3. Circular dichroism

As shown in Fig. 3 (curve a), the CD spectrum of the holoGAD in sodium phosphate buffer showed two negative bands between 200 and 228 nm, and two positive bands between 190 and 200 nm and 230–250 nm. Compared with the holoGAD, the negative bands in the apoGAD (Fig. 3, curve b) were slightly shifted to a lower wavelength; also its CD molar ellipticity decreased in the first negative band. On the contrary, the positive band at 232–250 nm moderately increased its CD molar ellipticity. These results corroborated that the secondary structure of the GAD had changed, but not significantly, when PLP was removed from the holoGAD. In fact, it was determined that the holoGAD had a higher β-sheet content than the apoGAD (39% versus 27%), whereas the former had a similar α-helix content to the latter (13% versus 14%). These findings further confirmed that a conformational change of the GAD had occurred during the conversion between the apoGAD and the holoGAD. Interestingly,

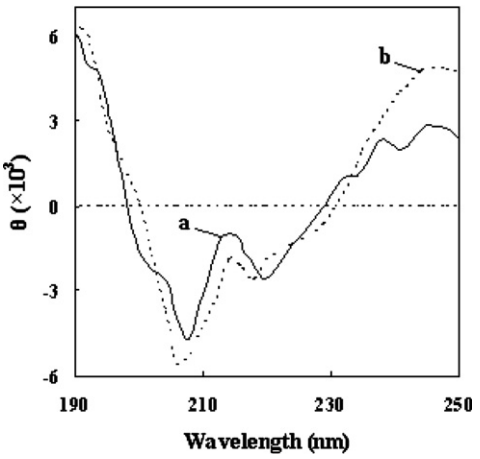

Fig. 3. Circular dichroism spectra of GAD. (a) holoGAD; (b) apoGAD. The concentration of GAD is 0.2 mg/ml in 50 mM sodium phosphate buffer, pH 5.6.

brain GAD holoGAD underwent great changes in both of its α-helix and β-sheet structures after conversion to the apoGAD (Chen et al., 1998). These differences can be attributed to the different molecular structures between rice germ GAD and brain GAD.

3.4. Chemical modifications

The PLP-dependent GAD from *E. coli* has a conserved lysine residue at its active site, which is subject to coenzyme binding and conformational changes (Tramonti et al., 2002). To the best of our knowledge, no previous research has been performed on chemical modifications of amino acids, except the lysine, in plant GAD. In order to gain more understanding of the rice germ GAD's catalytic function, several chemical reagents were selected to intentionally modify specific amino acids or functional groups, to reveal their underlying contributions. The effect of the reagents on GAD activity is shown in Table 1. Among the reagents used to chemically modify the GAD, only a few could inactivate the enzymatic activity by more than 10% after 30 min (Table 1). It appears that rice germ GAD was not significantly affected by the reagents selective against tryptophan, methionine, disulfide bond, hydroxyl and carboxyl groups, but was moderately inactivated (40%) by DEPC against histidine, and completely inhibited by BD against arginine. This indicates that the tryptophan, methionine residues and disulfide bond, hydroxyl and carboxyl groups neither exist in the GAD's active site, nor play critical roles nor make contributions to the enzymatic function.

DEPC is a common chemical modifier for histidine residue and has been widely used in enzyme modifications (Gacheru, Trackman, & Kagan, 1988; Swenson, Williams, & Massey, 1982; Vik & Hatefi, 1981). In the present work, the modification of the histidine residues in the enzyme was performed with different concentrations of DEPC. Fig. 4 shows that rice germ GAD was inactivated by DEPC in a dose-dependent way. Increasing the DEPC concentration decreased the remaining enzyme activity. When the DEPC concentration increased to 60 mM, the remaining enzyme activity approached to a minimum value of 60%. Meanwhile, the absorbance intensity of the modified enzyme at 280 nm also decreased when the DEPC concentration increased. Its profile was similar to that of the deceasing enzyme activity. This incomplete inactivation of GAD implied that the histidine residue was very possibly involved in the active site and played functional roles to a certain degree in the enzyme catalysis, but it was not the most critical residue to decide the fate of the enzymatic reaction.

Since the GAD's enzymatic activity could be completely inactivated after the arginine modification by BD, the effects of BD in terms of its concentration and the modification time were systematically investigated, with the results shown in Fig. 5. The linear semi-logarithmic plot of the remaining activity *vs* time (Fig. 5a) suggests that the enzyme inactivation is a simple chemical event and follows a pseudo-first order kinetics. Also, plotting the first-order rate constant (calculated from Fig. 5a) of the inactivation against the inhibitor's (BD) concentration (Fig. 5b) gave a straight line. The second-order rate constant for the inactivation was also calculated from the plot of the slopes. GAD inactivation by BD was highly significant as reflected by the second-order rate constant value of 109.6 M^{-1} min^{-1}. Plotting $\log 1/t_{1/2}$ against log (BD) resulted in a straight line (Fig. 5c) with a slope of 1.03, indicating that each active site in the enzyme holds one arginine residue and the modification of this arginine residue can significantly impair the GAD activity. Most likely, this arginine residue is at the substrate-binding site. Nevertheless, Fig. 6 shows that the substrate glutamate could partially protect the GAD from chemical modification by BD.

In summary, holoGAD rather than apoGAD gave a weak UV absorption at 420 nm. Also, holoGAD exhibited a strong peak at 308 nm and a weak peak at 336 nm in its

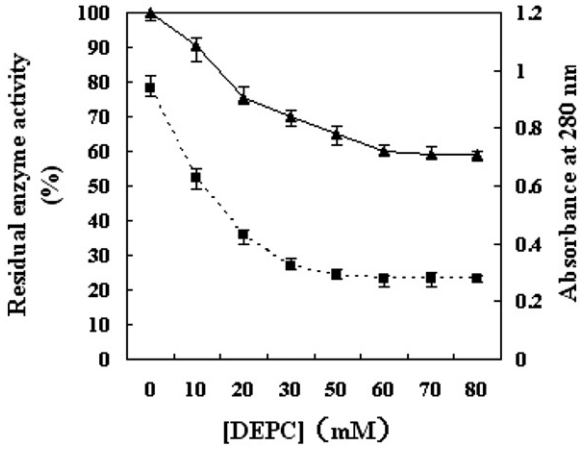

Fig. 4. Effects on the absorbance at 280 nm and enzyme activity of GAD by DEPC modification. (▲) Residual enzyme activity; (■) Absorbance at 280 nm ($n = 3$).

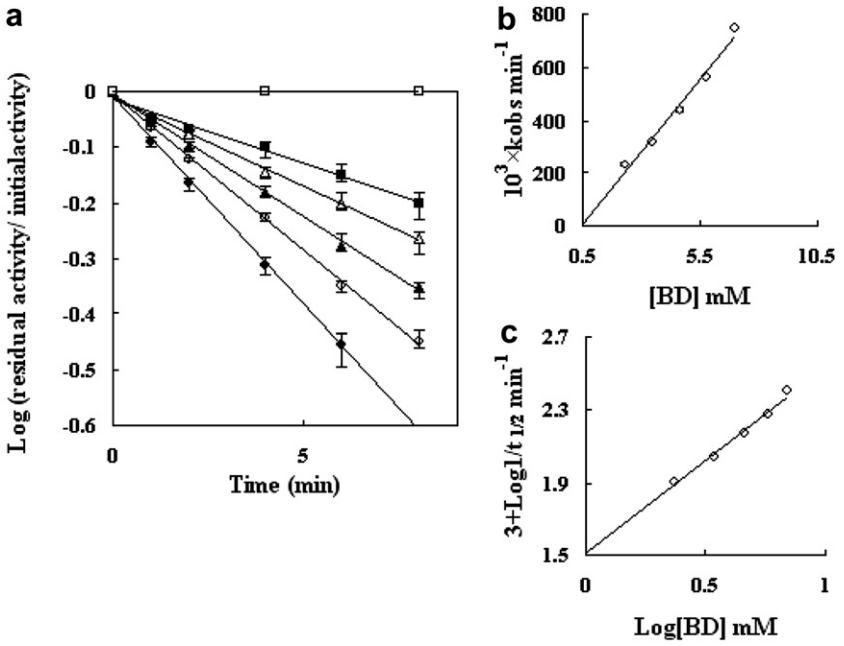

Fig. 5. (a) Kinetics of the inactivation of GAD by BD ($n = 3$). The concentrations of BD are 0 (□), 2.3 mM (■), 3.5 0mM (Δ), 4.7 mM (▲), 5.8 mM (○) and 6.9 mM (♦). (b) Plot of k_{obs}. determined from the slope of the inactivation kinetics, as a function of modifier concentration, to determine the second-order rate constant. (c) Plot of $\log(1/t_{1/2})$of inactivation against log (modifier concentration), to determine the reaction order.

fluorescence emission spectrum. However, apoGAD led to a 20% increase in the fluorescence emission at 308 nm. Investigations of the secondary structural elements of holo-GAD and apoGAD revealed that the former had a greater β-sheet content than the latter (39% versus 27%), whereas both had a similar α-helix content (13% versus 14%). These findings confirmed that a slight conformational change had occurred when PLP bound to apoGAD to form the holo-GAD. Chemical modifications of the GAD by some selected reagents indicated that histidine residue(s) in the enzyme played certain roles, but its requirement was not obligatory for the enzyme activity. The study also suggested there was one arginine residue in the GAD active site, and most likely at the substrate-binding site.

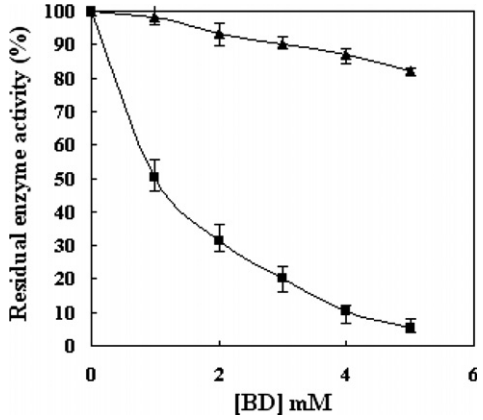

Fig. 6. Substrate protection on BD modification. The enzyme was mixed with different concentrations of BD in 100 mM MES buffer, pH 5.0 in the presence of 10 M glutamate (▲) and absence of glutamate (■) ($n = 3$).

Considering the fact that GABA is an important bioactive regulator in biological systems and its biosynthesis is through the α-decarboxylation of glutamate by GAD in a rate-limiting step, this study on characterisation of the molecular properties of the rice germ GAD is deemed to be necessary and useful, especially when GABA is produced with aid of the rice germ GAD. Although other molecular properties and mode of function of rice germ GAD need further exploration, we expect our current work will be helpful for developing a GABA-based, cost-effective rice germ GAD-related functional food as a dietary supplement and/or nutraceutical against hypertension (Hayakawa et al., 2004), sleeplessness, depression and autonomic disorder (Tadashi et al., 2000).

Acknowledgments

The authors deeply appreciate the generosity of Shanghai Store & Transport of Grain Co., Ltd. for its gift of rice germ and want to extend our thanks to the Testing and Analysis Center of Southern Yangtze University for its technical help on sample analysis. This project was financially supported by the National Natural Science Foundation of China (Project number 20671044).

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