Glutamate Decarboxylase (GAD) Extracted from Germinated Rice: Enzymatic Properties and Its Application in Soymilk

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Summary Glutamate decarboxylase (GAD) is an important enzyme in biological metabolisms acting on catalyzing the irreversible α-decarboxylation of L-glutamic acid to γ-aminobutyric acid (GABA) and CO2, which was focused in this study. Three rice varieties different in color were germinated at different times and used for crude GAD extraction. Crude GADs with an optimal germination time from germinated black (GBR), red (GRR), and white (GWR) rice were evaluated for enzymatic properties, including the effect of pHs, temperatures, and concentrations of both L-glutamic acid and pyridoxal 5′-phosphate (PLP).

Crude GAD with optimum enzymatic properties was selected to be partially purified using ammonium sulfate (AMS) precipitation. The obtained GAD was supplemented to soymilk and determined for GABA content. All crude GADs from germinated rice at 10 germination days presented the highest enzyme activity. For enzymatic properties, crude GADs showed the highest activity at pH in a range of 5.6–6.0 at 60°C. The Km values of crude GADs were in the range of 7.68–8.06 mM for L-glutamic acid and 0.15–0.20 μM for PLP and were the lowest in crude GAD from GBR. GAD from GBR presented the highest enzyme activity in the fraction with 50% saturation (v/v) after AMS precipitation and it was purified for 14.61 folds. The addition of this GAD (1.0%, v/v) resulted in the increasing of GABA content in soymilk to 53.79 mg/100 mL, accounted for 1.23 times compared with control.

Key Words glutamate decarboxylase, germinated rice, enzymatic properties, γ-aminobutyric acid, soymilk

Glutamate decarboxylase (GAD) [EC 4.1.1.15] is a pyridoxal 5′-phosphate (PLP) dependent enzyme that catalyzes the conversion of L-glutamic acid to γ-aminobutyric acid (GABA) and CO2 by the irreversible α-decarboxylation (1). Previously, GAD has been extracted and purified from several origins, such as microorganisms, animals, and plants. However, their enzymatic properties were different depending on the nature of GAD origins. For example, the optimal pH was in the range of 4.0–6.0 for bacterial GADs (2–5), 6.8–7.0 for animal GADs (6, 7), and 5.5–6.0 for plant GADs (8–10), and The optimal temperature of GAD was varied between 37–40°C in most plant GADs (8–11), while GAD from wheat seedling (12) and squash (13) showed the optimal temperature at 55 and 60°C, respectively.

GAD is responsible for the increase of GABA in germinated cereal grains, especially germinated rice (14). Germinated rice is also considered as functional food because it contains more both basic nutritional components and bioactive compounds, such as linoleic acid, tocotrienols, and γ-oryzanol than those in brown rice (15). Because GABA is a common neurotransmitter in the brain used as a medicinal supplement to help reduce anxiety in some people (16), therefore several studies have attempted to enhance GABA in many food products, such as beverages (17), breakfast cereals (18), bread (19, 20), noodles (21), and yogurt (22), through various application methods, including supplementation, fermentation, and bioconversion.

In this study, crude GADs were extracted from different germinated rice whether black, red, and white rice to study the changes during germination process. Crude enzymes were also determined for enzymatic properties, including the effect of pH and temperature and Km values for substrate and coenzyme. The suitable enzyme was selected for partial purification using ammonium sulfate (AMS) precipitation and application in soymilk product. This is also the first time to apply GAD from germinated rice in foods.

Materials and Methods

Materials. Three varieties of paddy rice (O. sativa L.) samples, including Hom Deang Sukhothai 1 (red rice; RR), Hom Dam Sukhothai 2 (black rice; BR), and Hom Mali 105 (white rice; WR) were obtained from the Organic Agriculture Project, Sukhothai Airport, Thailand. GABA, L-Glutamic acid, PLP, and phenylmethyl-
sulfonylfluoride (PMSF) were purchased from Sigma (St Louis, MO, USA). Acetoniitrile was of high-performance liquid chromatography (HPLC) grade. All other chemicals and reagents used were of analytical grade.

Preparation of germinated rice. Paddy rice samples (300 g) were soaked in water at a ratio of 1:5 (w/v) at room temperature. The water was changed every 8 h and then decanted after 24 h. The soaked samples were wrapped by cheesecloth and covered by the black plastic bag. At the initial stage of germination, the samples were allowed to germinate for 3 d until the bud has appeared. The germinated samples were separately placed in 15 plastic boxes (10×15×5 cm) containing the wet cotton (approx. 1 cm thickness) and one layer of tissue paper at the top. The water was put into the boxes (approx. 2 cm height) to grow the samples during a period of germination at room temperature. Germinated samples were daily collected from one box per germination day until 15 d. The fresh samples were stored at −80°C and used for GABA content and GAD activity analysis.

Determination of GABA content. The derivatization of GABA was conducted prior to the analysis of GABA content following the modified method described by Khuhawar and Rajper (23). The analysis of GABA content was carried out by HPLC according to the modified method of Jannoey et al. (24) using an HPLC (Shimadzu, Kyoto, Japan) system equipped with a pump (LC-10ADVP), photodiode array detector (SPD-10AVP), and Shimadzu Class-VP software. The separation of derivatized GABA was performed on a reverse phase HPLC column (4.6×250 mm, Inertsil ODS-3, 5 μm; GL Sciences Inc., Tokyo, Japan) system at 40°C. A combination of mobile phase containing acetonitrile (mobile phase A) and 0.1% formic acid (mobile phase B) was applied. The gradient elution conditions for mobile phase A were initially at 35%; 40% at 5 min; 55% at 10 min; and 35% at 20 min. The flow rate was maintained at 1.0 mL/min and the wavelength was set at 330 nm throughout the analysis. GABA content was detected using an external standard method.

Preparation of crude GAD. One gram of fresh sample was randomly selected, frozen in liquid nitrogen, and immediately ground using mortar. Five milliliters of 50 mM sodium phosphate buffer (pH 5.6), containing 2 mM 2-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM PMSF was added to a ground sample. The mixture was poured into a centrifuge tube and then centrifuged at 10,000×g at 4°C for 20 min (11). The supernatant was collected as crude GAD and immediately used for analysis.

Determination of GAD activity. The enzyme activity was performed using the modified procedure described by Zhang et al. (11). The reaction mixture consisted of 600 μL 50 mM sodium phosphate buffer (pH 5.6), containing 100 mM L-glutamic acid and 0.2 mM PLP, and 200 μL crude GAD. The reaction solution was incubated at 40°C for 60 min and immediately inactivated by 2-hydroxynaphthaldehyde (HN) derivatization (23). The solution was filtered through a 0.45-μm membrane filter. The filtrate was analyzed for GABA content using HPLC as described above. Total activity (U) is one unit of GAD activity that releases 1 μmol of GABA produced from L-glutamic acid within 30 min at 40°C, while specific activity is defined as units of GAD activity per mg of total protein.

Protein assay. Protein concentrations were analyzed by the method of Bradford (25) using bovine serum albumin as standard.

Effect of pH on enzyme activity. The enzyme assay was performed at 40°C and different pH buffers ranging from pH 3.0 to pH 8.0 using two buffer systems (50 mM citrate buffer, pH 3.0–4.0 and 50 mM sodium phosphate buffer, pH 5.0–8.0). The enzyme activity was measured as those described above.

Effect of temperature on enzyme activity. GAD activity was determined by carrying out enzyme assays at pH 5.6 and temperatures ranging from 30 to 80°C. The enzyme activity was measured as those described above.

Determination of Km values. L-Glutamic acid, a substrate for GABA production catalyzed by GAD was evaluated for Km values for each GAD enzyme at pH 5.6 and temperatures ranging from 1.25–25 mM. A coenzyme PLP at various concentrations (0.05–100 μM) was also performed in enzyme assays to determine Km values. The Km values of L-glutamic acid and PLP for each GAD were determined from the Lineweaver-Burk plot.

AMS precipitation. One gram of dried germinated rice was blended with 50 mL of 50 mM sodium phosphate buffer (pH 5.6), containing 2 mM 2-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF. The mixture was filtered under the vacuum. The filtrate was centrifuged at 10,000×g at 4°C for 20 min. The supernatant was collected as crude GAD and immediately used for AMS precipitation (8). The crude enzyme was precipitated by slowly adding solid AMS to 10, 30, 50, 70, and 90% (v/v) saturation. After centrifugation, the pellets from each% saturation were dissolved in 50 mM phosphate buffer (pH 5.6) and dialyzed at 4°C. The obtained enzyme was further used for determination of GAD activity and total protein content as those described above. Both 1 mM of PLP and 1 mM of PMSF were added to the buffer to stabilize the GAD and prevent proteolytic degradation.

The application of GAD in soymilk. Soymilk production was conducted according to the modified method of Odou and Egbo (26). For the GAD application, GAD was added to soymilk at 0.1, 0.5, 1.0, and 2.0% (v/v). Soymilk containing GAD and control (0% GAD) were then incubated at 60°C for 30 min to perform the reaction of GAD activity. The mixture was pasteurized at 80°C for 20 min and stirred regularly to avoid burning. Soymilk was immediately cooled and stored at 4°C in a refrigerator until used for determination of GABA content as described above.

Statistical analysis. All experiments were expressed as mean and standard deviation (n = 3). One-way analysis of variance (ANOVA) and Duncan’s new multiple range tests (DMRT) for multiple comparisons were performed using SPSS software (SPSS Inc., Chicago, IL, USA) (p<0.05).
Results

GAD activity in germinated rice during different germination times

Our preliminary study indicated that the sprout of germinated rice contained the highest GAD activity (data not shown), thus this part from each sample was selected to determine the optimal time for GAD activity during germination. A trend of GAD activity in all varieties of germinated rice was increased at the initial period until highest in a moment and decreasing later (Fig. 1). Germination at 8–13 d for GBR, 9–13 d for GRR, and 10–13 d for GWR exhibited a high level of GAD activity (p<0.05). The highest GAD activity was presented at 10 germination days for all crude GADs from germinated rice, thus germination at 10 d was the optimal time to germinate rice samples for the study of enzymatic properties.

Enzymatic properties of crude GADs from germinated rice

The effect of pH on the enzyme activity of GAD from germinated rice was conducted at pH 3.0–8.0 using L-glutamic acid as substrate as shown in Fig. 2A. At the pH range 5.6–6.0, high activity was exhibited in crude GAD from GBR, while crude GADs from GRR and GWR exhibited high activity at pH 5.0–6.0 (p<0.05). In addition, the enzyme activity of crude GADs was tested at a different temperature, ranging from 30–80˚C to determine the optimal temperature (Fig. 2B). The highest activity was shown at 60˚C for all crude GADs (p<0.05). This result suggested that the pH range 5.6–6.0 at 60˚C was optimal pH and temperature for crude GADs from germinated rice.

The kinetic parameters, Km values for L-glutamic acid and PLP of crude GAD from different germinated rice varieties were evaluated in this study and presented in Table 1. The highest Km value for L-glutamic acid was presented in crude GAD from GRR, followed by those in GWR and GBR, respectively. Furthermore, crude GAD from GWR exhibited the highest Km for PLP, followed by those in GRR and GBR, respectively. Because Km is the substrate concentration that is required for the reaction to achieve half of the maximum velocity (Vmax), thus crude GAD from GBR that exhibited the lowest Km values for both L-glutamic acid (7.68 mM) and PLP (0.15 μM) was selected for the precipitation using AMS and further application in soymilk product.

Partial purification using AMS precipitation

Crude GAD extracted from GBR was partially purified using AMS precipitation at different percentage saturation (%v/v) and the result was shown in Fig. 3. The highest specific activity of GAD was obtained from the fraction with 50% saturation (4.48 U/mg), followed by the
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The result of the current study showed that GAD activity in germinated rice has changed during the germination and related to L-glutamic acid in rice samples. The level of L-glutamic acid was correlated with GABA content, because it is a substrate for GABA production via α-decarboxylation catalyzed by GAD enzyme (27). A previous study also reported that GABA accumulation and GAD activity in germinated rice is affected by the initial concentrations of L-glutamic acid (28, 29).

For enzymatic properties, the optimal pH of crude GADs from germinated rice was in the range of 5.6–6.0, which was in agreement with those of GAD from cowpea (30), potato (10), rice germ (11), wheat seedling (12), and squash (13). The optimal temperature of this crude GADs was similar to those of GADs from Aspergillus oryzae (2) and squash (13) that was optimal at 60°C. The Km values that are the enzyme kinetic parameter obtained from the Lineweaver-Burk plots were evaluated in this study. The Km values for L-glutamic acid of crude GADs from germinated rice were closed to those values in GAD from squash (8.3 mm, 13), while these values were lower than those Km values of GAD from rice bran (27.4 mm, 8) and rice germ (32.3 mm, 11). In addition, Km values for PLP of these crude GADs were similar to those Km values for GAD from human brain (0.13 μM, 6). However, the Km value of other GADs for PLP was also varied, such as 0.04 μM for Neurospora crassa conidia (34), 0.05 μM for mouse brain (32), 1.16 μM for rice bran (8), 1.70 μM for rice germ (11), and 2.00 μM for potato (10). This result indicated that the variation of enzymatic properties depended on the origins of GAD enzyme and GAD from germinated rice has a potential for applications at wide acidic pH ranges with warm temperature.

Partial purification using AMS precipitation was performed to concentrate the enzyme. The previous study by Wang et al. (8) reported the precipitation using 30–50% (v/v) saturation of AMS was proper for GAD from rice bran, which was in agreement with the result in this study. GAD from GBR was purified for 14.61 folds after AMS precipitation, which was higher than the purification fold in other GADs from plants, such as rice bran (4.60 folds, 8) and germinated faba bean (5.63 folds, 9).

Soy milk was selected to study the GAD application because soybean is a rich source of L-glutamic acid, which is the substrate for GABA production by the α-decarboxylation of GAD enzyme. The increase of GABA content in soymilk was due to the decarboxylation of glutamic acid catalyzed by GAD enzyme. This phenomenon was corresponded with the GABA enrichment by GAD supplementation in breakfast cereals and wheat flour bread (18, 20).

In conclusion, germinated rice was suggested as the important source of GAD enzyme and the enzyme activity was increased during germination. Enzymatic properties of GAD in this study demonstrated that it is a potential enzyme to increase GABA content in soymilk based on optimal condition. This study may lead to the guideline of the application of GAD to produce GABA enriched food. However, other properties of the final product are required and should be considered.

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Table 2. Partial purification of GAD from GBR.

| GAD fractions | Total protein (mg/mL) | Total activity (U) | Specific activity (U/mg) | Purification fold | Recovery (%) |
|---------------|-----------------------|--------------------|--------------------------|------------------|-------------|
| C. GAD        | 49.52                 | 19.72              | 0.40                     | 1.00             | 100.00      |
| GAD*          | 2.50                  | 14.52              | 5.82                     | 14.61            | 73.67       |

Note: C. GAD=Crude GAD, GAD*=GAD derived from 50% AMS precipitation and dialysis.

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Fig. 4. GABA content of soymilk supplemented with GAD from GBR. Different letters on bars indicate significant differences (p<0.05).

fraction with 30% saturation (0.54 U/mg) (p<0.05). The enzyme activity was not found in the fractions with 10 and 90% saturation (p<0.05).

The potential of partial purification step was determined after dialysis using a standard buffer (pH 5.6) at 4°C for 6 h. Total protein, total activity, specific activity, purification fold, and recovery (%) of GAD from the fraction with 50% AMS were performed and presented in Table 2. After precipitation using AMS 50% saturation and dialysis, total protein and total activity of GAD were decreased, while specific activity was increased from 0.40 to 5.82 U/mg. In addition, GAD from GBR was purified for 14.61 folds and recovered for 73.67%.

GAD application in soymilk product

The concentrate of GAD was varied at 0.1 to 2.0% (v/v) and applied to soymilk during a production process. GABA content in soymilk supplemented with GAD was measured and showed in Fig. 4. GABA content was increased in soymilk supplemented with GAD from 0.1% to 1.0% (v/v) and decreased at the supplementation at 2.0%. The highest GABA content was presented in soymilk supplemented with 1.0% GAD (53.79 mg/100 mL), however, it was not significantly different from those supplemented with 0.5% GAD (p>0.05). Additionally, there was no significant difference between GABA content in control and soymilk supplemented with 0.1 and 2.0% GAD.

Discussion

The result of the current study showed that GAD activity in germinated rice has changed during the germination and related to L-glutamic acid in rice samples. The level of L-glutamic acid was correlated with GABA content, because it is a substrate for GABA production via α-decarboxylation catalyzed by GAD enzyme (27). A previous study also reported that GABA accumulation and GAD activity in germinated rice is affected by the initial concentrations of L-glutamic acid (28, 29).

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Disclosure of State of COI

All authors have no conflicts of interest to declare.

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